The role of PRAS40 in insulin action : at the intersection of protein kinase B (PKB/Akt) and mamalian target of rapamyein (mTOR)

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Chapter 7

General discussion

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Proper glucose homeostasis is important for the balanced use of energy sources, such as carbohydrate, amino acids and fatty acids by the human body. In addition, high circulating glucose concentrations are unfavourable because of the chemical reactivity of glucose with amino-groups in proteins. Insulin plays a critical role in maintaining adequate glucose levels. The intermediates involved in insulin signalling within cells are numerous and their specific role is not always clearly defined Within the insulin signalling cascade several nodes are present: the insulin receptor (IR), insulin receptor substrate (IRS) proteins, phosphatidyl inositol 3'-kinase (PI3K), protein kinase B (PKB/Akt), mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase /ERK kinase (MEK) (more detail in reviews (1;2)). The interplay of these different nodes are responsible for proper insulin action. Our research focus was on phosphorylation substrates of protein kinase PKB/Akt . This protein kinase is able to phosphorylate Ser/Thr residues in downstream targets which possess the Arg-Xaa-Arg-Xaa-Xaa-pSer/pThr motif.(3;4). Through phosphorylation of these downstream targets, PKB/Akt is able to regulate multiple cellular processes, like glucose homeostasis, cell growth, apoptosis and protein synthesis. The scope of the presented research in this thesis was:

1) to determine physiological PKB/Akt substrates in target tissues for insulin action;

2) to examine alterations in expression, phosphorylation status and localisation of these substrates in association with conditions of insulin resistance;

3) to search for a function of these PKB/Akt substrates.

PKB/Akt substrates AS160, NDRG2 and PRAS40

With the use of the antibody recognizing the Arg-Xaa-Arg-Xaa-Xaa-pSer/pThr motif, we were able to study multiple PKB/Akt substrates. Increased phosphorylation of PKB/Akt substrate of 160 kDa (AS160) on residue Thr642 was detected in insulin stimulated rat liver, skeletal muscle, cardiac muscle and adipose tissue (chapter 3). Increased phosphorylation of AS160 was observed in biopsies from human skeletal muscle under conditions of hyperinsulinaemia. When the individual exhibited insulin resistance phosphorylation of AS160 was impaired in human skeletal muscle (5). The function of AS160 has been linked to GLUT4-mediated glucose uptake in skeletal muscle and adipose tissue (6-11). The Rab GTPase protein domain present in the AS160 protein has been found to be of critical importance for proper glucose uptake.

As putative PKB/Akt substrate, we also studied N-myc downstream-regulated gene 2 (NDRG2). The NDRG family consists of four genes (NDRG1-4). Residue Thr348 of NDRG2 matched the RxRxxpS/pT motif which PKB/Akt employs to phosphorylate downstream targets (12). In heart and skeletal muscle from insulin-stimulated rats, phosphorylation of NDRG2-Thr348 was increased. In liver, skeletal muscle and adipose tissue from insulin-stimulated mice, phosphorylation of NDRG2-Thr348 was increased. However different protein kinases like protein kinase C and SGK1, which are not directly linked to the insulin signalling pathway, are involved in phosphorylating NDRG2 (12;13). Further research to examine the role of NDRG2 in insulin signalling will provide us with a better understanding of the insulin signalling cascade. Animal models for insulin resistance or analysis of human biopsies comparing healthy subject to diabetic subjects could be an approach to answer these questions. More research has been conducted on NDRG2 in respect to cancer, due to the link with the oncogene myc (14).

The most prominent substrate for PKB/Akt was found to be PRAS40. Since not much was known about the function of the PRAS40 protein, we decided to dedicate further research to this particular protein.

PRAS40 as physiological PKB/Akt substrate in insulin target tissues

Initially PRAS40 was described as a PKB/Akt substrate with a phosphorylation site at residue Thr246 (15). In chapter 3 we describe more in detail both *in vitro* and *in vivo* phosphorylation of PRAS40 on Thr246. *In vitro* phosphorylation is markedly diminished after incubation with the PI3K-inhibitor wortmannin. In E2 cardiomyocytes, phosphorylation of PRAS40-Thr246 is completely abolished while in A14 fibroblasts and L6 myotubes phosphorylation is reduced (chapters 3 & 4). These findings indicate that next to PKB/Akt other kinases are able to phosphorylate PRAS40 on Thr246. In agreement with this, PKB/Akt deficient cells showed a marked reduction in PRAS40-Thr246 phosphorylation however not a complete abolishment of phosphorylation (15). We should

keep in mind that the used PKB/Akt-deficient cells were deficient for PKBα/Akt1 and PKBβ/Akt2, however, not for PKBγ/Akt3. PKBγ/Akt3 has been identified as PRAS40-Thr246 phosphorylation kinase in tumorigenesis (16). However, how this relates to insulin signalling remains unknown. Next to PKB/Akt, the Pim kinase has been proposed as upstream phosphorylation kinase for PRAS40 on Thr246 (17). Also in the insulin producing Ins1E cells, an increase in PRAS40-Thr246 phosphorylation was detected after insulin stimulation (Fig. 1). In these cells, phosphorylation was PI3K dependent, since wortmannin was able to inhibit PRAS40-Thr246 phosphorylation. Why differences in phosphorylation are detected among cell types remains unclear. However, this indicates the importance of PRAS40 in insulin sensitive tissues.

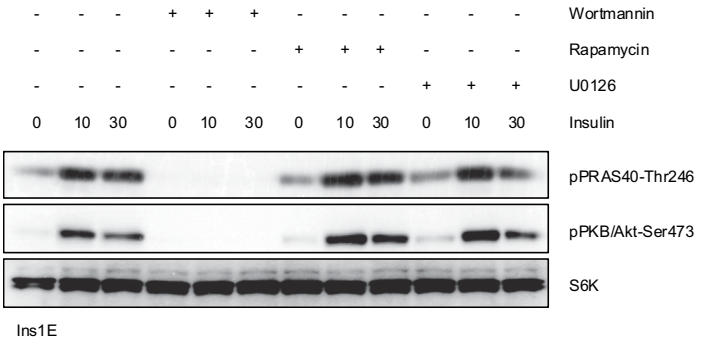

Figure 1. *PRAS40-Thr246 phosphorylation in Ins1E cells is PI3K dependent. Ins1E cells were stimulated with insulin during the indicated times (min). Where indicated cells were pretreated with wortmannin (PI3K inhibitor), rapamycin (mTOR inhibitor) or U0126 (MEK inhibitor). Samples were analyzed using Western Blotting techniques.*

In chapter 4 we examined phosphorylation on Ser183, being another site within the PRAS40 protein which is under control of both PI3K and mTORC1. Reports in the literature only state the phosphorylation of Ser183 to be mTORC1 dependent (18-20). In these articles the PRAS40 protein was ectopically expressed and phosphorylation was studied using radiolabeled ATP. By introducing mutations in the PRAS40 protein we were able to establish the importance of the Thr246 phosphorylation site and the mTORC1-binding site for proper phosphorylation of PRAS40 on Ser183 (chapter 4).

PRAS40 phosphorylation under conditions of insulin resistance

A clear link is present between i*n vivo* phosphorylation of PRAS40-Thr246 and insulin sensitivity. In chapter 3 we describe that phosphorylation of PRAS40-Thr246 is reduced in the insulin resistant state. On the other hand when examining human subjects who were better able to control their glucose levels because of high insulin sensitivity, we observed that these subjects displayed an increased phosphorylation of PRAS40-Thr246 protein in

skeletal muscle (21). These data suggested that PRAS40 phosphorylation may serve as a marker for insulin sensitivity.

To validate this hypothesis we analysed by western blots the level of PRAS40-Thr246 phosphorylation leukocytes from blood from diabetic patients. After comparison of two different patients, the phospho-specific PRAS40-Thr246 antibody displayed an additional band in the test subject who only received metformin and not in the test subject who received a combination of metformin and insulin glargin (Fig. 2). The subject on metformin was more insulin sensitive compared to the subject using a combination of metformin and insulin glargin. This might indicate that a decrease in insulin sensitivity might account for the loss of the additional band. With the use of a 2D approach and mass spectometry, the identity of the band can be determined. Isolation of leukocytes for protein analysis requires a specific approach, which should also be optimized prior to further research. This optimisation will increase the reproducibility of the experiments with human samples. Optimisation should include the verification whether only leukocytes have been isolated. With the use of flow cytometry or western blotting, the leukocyte surface marker CD45 can be determined to exclude the presence of other blood components. For example via a similar approach, detection of anion exchanger 1 will indicate the unwanted presence of erythrocytes in the sample. Preliminary results from isolated human leukocytes show that under conditions of hyperinsulinaemia, PRAS40-Thr246 phosphorylation is increased in time (data not shown). Further preliminary results show a correlation between PRAS40-Thr246 phosphorylation and insulin sensitivity in human subjects.

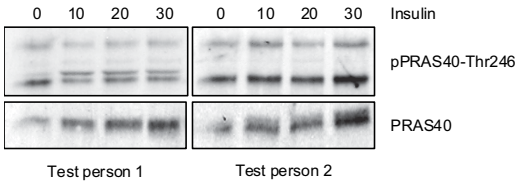

Figure 2. *Detection of pPRAS40-Thr246 in isolated human leukocytes. Test person received a hyperinsulinaemic euglycaemic clamp. During hyperinsulinaemia blood was isolated at the indicated time point (in min) after start of insulin infusion. Leukocytes were isolated and analyzed using Western blotting techniques. Test peson 1 received metformin, while test person 2 received a combination of metformin and insulin glargine.*

Function of PRAS40 in (insulin) signal transduction

In chapter 5 we describe the presence of a nuclear export signal in the PRAS40 protein. Next to the phosphorylation sites, we now have a functional domain, which has as function to export PRAS40 from the nucleus. Opposite from export, there is import, however, how import of PRAS40 is mediated remains unknown. 14-3-3 proteins are known chaperones and could be chaperons for PRAS40 to mediate nuclear import. Experiments using 14-3-3 fusion proteins showed an interaction of PRAS40 with 14-3-3 only after insulin stimulation (Fig. 3). Mutation of the nuclear export signal was able to sensitize insulin-stimulated PKB/Akt and S6K phosphorylation (chapter 5). In parallel mutation of the nuclear export signal in PRAS40 resulted in increased 14-3-3 binding under basal conditions and under insulin-stimulated conditions. Further research will provide more insights into the role of PRAS40 in the nucleus, how import of the protein is mediated and whether nuclear import of PRAS40 is 14-3-3 dependent. By creating a fusion protein composed of PRAS40 and a fluorescent protein like green fluorescent protein (GFP), experiments could be carried out following real-time translocation of the PRAS40 protein to the nucleus. Furthermore these experiments can be repeated in cells which have modified 14-3-3 levels in order to establish whether observed differences are thus 14-3-3 dependent. Another approach could be to create a 14-3-3 fusion protein with another fluorescent protein like yellow fluorescent protein (YFP). The combination of the 14-3-3 fusion protein and PRAS40 fusion protein can be followed in real-time to observe whether the fusion proteins are in proximity of each other.

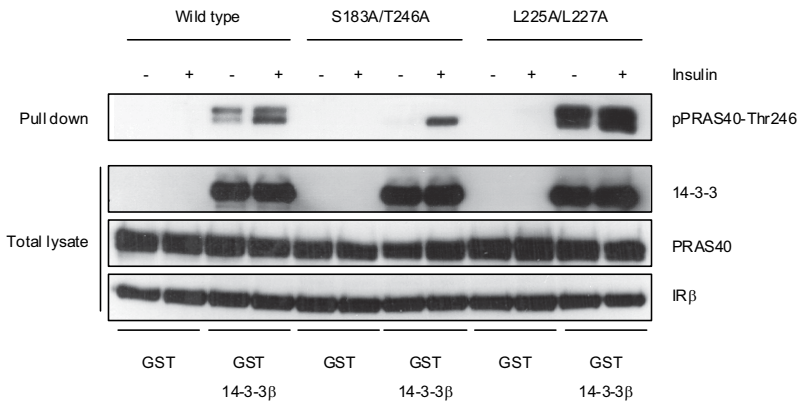

Figure 3. *PRAS40 binding to 14-3-3. A14 cells transfected with plasmids expressing wild type or mutant S183A/T246A-hPRAS40or L225A/L227A-hPRAS40. After insulin stimulation cells were lysed. GST pull down was performed using coupled GST or GST fusion protein to 14-3-3β. Pull down and total lysates were analyzed using Western blotting techniques.*

Chapter 6 describes the effects of variations in PRAS40 levels under normal conditions and under conditions of palmitate-induced insulin resistance. In parallel, we tried to establish a role for PRAS40 in palmitate-induced reactive oxygen species (ROS) production. The rationale behind this hypothesis is derived from the fact that increased PRAS40-Thr246 phosphorylation is associated with increased survival of motor neurons after spinal cord injury (22). PRAS40-Thr246 phosphorylation was even further increased in copper/zinc-superoxide dismutase (SOD1) transgenic animals (23). The superoxide dismutases are a first line of defence against superoxide produced as a byproduct of oxidative phosphorylation. Mammals express three different SODs: SOD1, SOD2 and extracellular SOD. From data presented in chapter 6 we have seen that palmitate induces insulin resistance. Incubation of beta cells, cardiomyocytes and isolated mitochondria with the monounsaturated free fatty acid oleate resulted in increased levels of ROS (24). In line with ROS production, oleate increases mRNA of SOD1 to counteract the production of ROS (25). Therefore we considered the possibility that PRAS40 has a role in palmitate-induced ROS production. Unfortunately knockdown of PRAS40 made the measurements for ROS using a fluorescent probe highly irreproducible. Time did not allow us to measure ROS production by other means in these experiments. So it still remains to be determined whether the induction of insulin resistance by fatty acids involves steps involving ROS production through a PRAS40-dependent pathway.

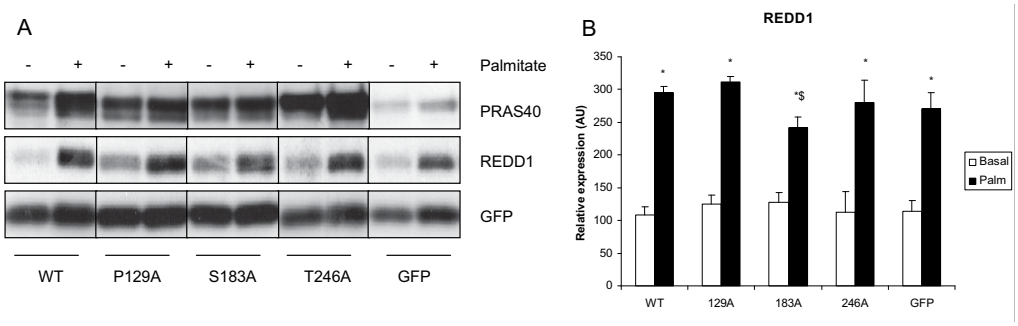

***Figure 4**. Reduced induction of REDD1 protein after palmitate treatment in cells expressing mutant S183A-hPRAS40. A. A14 cells were infected with plasmids expressing wild type hPRAS40 (WT) or mutant P129A-hPRAS40 (P129A), S183A-hPRAS40 (S183A) or T246A-hPRAS40 (T246A). Control cells were infected with empty vector only expressing GFP. Cells were treated overnight with 0.75 mM palmitate and samples were analyzed using Western blotting techniques. B. Quantification of REDD1 blots obtained in panel A. *, $p<0.05$ compared to basal; $, $p<0.05$ compared to WT.*

In addition to the previously mentioned experiments in relation to the mechanism of palmitate-induced insulin resistance we examined other signalling intermediates regulating mTORC1 activity. Regulated in development and DNA damage responses 1 (REDD1) is a

14-3-3 binding protein which results in release of TSC2 and via this mechanism REDD1 is able to downregulate mTORC1 (26;27). After palmitate treatment we observed a significant increase in REDD1 expression. REDD1 expression correlated with decreased mTORC1 signalling created by palmitate-induced insulin resistance. Expression of wild type hPRAS40 had no effect on the palmitate-induced increase in REDD1 protein expression. However, expression of mutant Ser183Ala-hPRAS40 resulted in an attenuation of the palmitate-induced increased expression of REDD1 (Fig. 4). This indicates that the Ser183 of PRAS40 mediates palmitate-induced REDD1 expression. More research on REDD1 expression, the link with the insulin signalling pathway and PRAS40 will clarify the importance of these three components in palmitate-induced insulin resistance

To further investigate the role of PRAS40 we also examined endothelial cells. Endothelial cells form the lining of blood vessels. The blood vessels ensure adequate supply of nutrients and oxygen to the organs of the human body. In the untreated diabetic patient, the blood vessels are continuously under stress due to exposure to chronically elevated plasma glucose levels, resulting increased risk for heart disease, diabetic retinopathy and impaired wound healing. Vascular endothelial growth factor (VEGF) is an important growth factor for endothelial cells. Especially when hypoxia develops within a tumour, VEGF is locally released inducing *de novo* vascularisation to supply the growing tumour with oxygen and nutrients. VEGF induces cell proliferation, migration, survival and vascular permeability via binding to the VEGF type 2 receptor and subsequent activation of PKB/Akt (28). PKB/Akt- and mTOR-activity are downregulated in human malignancies and metabolic disease (29-33). Hypoxia inducible factor 1α (HIF1α) expression is regulated by PI3K and mTOR. The stability of HIF1α is under control of oxygen and a drop in oxygen levels increases HIF1α levels. HIF1α has as downstream target VEGF, therefore it makes sense that certain tumours have elevated levels of HIF1α to ensure nutrient supply through vasculature to the inner tumour core.
We thus wondered whether PRAS40 is expressed in endothelial cells and whether the protein plays a role in VEGF mediated signalling. PRAS40 phosphorylation was detected on Thr246 and Ser183 in murine b.End3 cells. Stimulation of b.End3 cells with VEGF did not increase PRAS40-Thr246 or PRAS40-Ser183 phosphorylation (Fig. 5). As readout for VEGF action we saw an increase in pERK1/2-Thr202/Tyr204 after VEGF stimulation. As seen in previous described cell lines, also in b.End3 cells phosphorylation of PRAS40-Thr246 was reduced after wortmannin treatment and PRAS40-Ser183 phosphorylation was reduced after either wortmannin or rapamycin treatment. Unfortunately we were unable to detect an increase in PKB/Akt phosphorylation after VEGF stimulation in b.End3 cells, however, phosphorylation of mTOR downstream target S6K was prominent. Similar results were obtained in HUVECs and ECRFs. Next we examined the role of PRAS40 in HIF1α

and VEGF expression. First results displayed an increase in HIF1α and VEGF mRNA after PRAS40 knockdown. In the supernatant of the b.End3 cells in which PRAS40 was knocked down, VEGF protein levels showed a slight increase (data not shown). Further experiments will elucidate the impact of PRAS40 in VEGF mediated signalling. Endothelial cells provide a perfect cell system to study angiogenic properties. By modulating PRAS40 protein levels through either knockdown or overexpression, endothelial cells can be used to study angiogenesis by seeding the cells into a matrigel matrix. In this matrix the endothelial cells can create tube like structures which will provide information on the role of PRAS40 in the process of angiogenesis/VEGF mediated signalling.

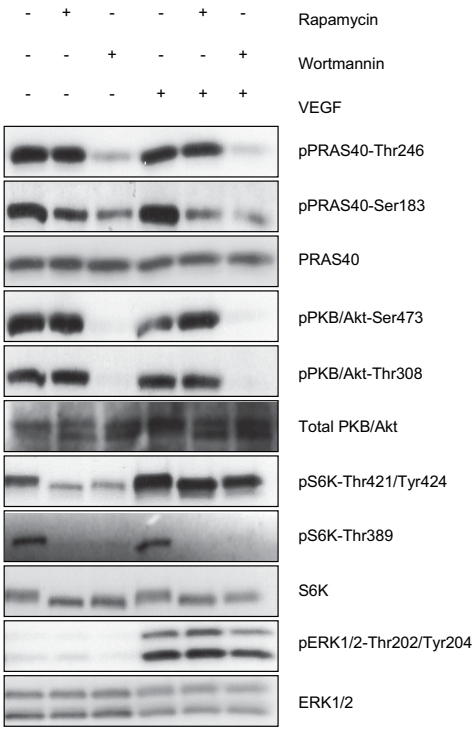

Figure 5. *PRAS40-Thr246 is regulated by PI3K, while PRAS40-Ser183 phosphorylation is regulated by PI3K and mTORC1 in endothelial cells. b.End3 cells were serum-starved and subsequently pretreated with wortmannin (100 nM, 10 min) or rapamycin (100 nM, 15 min). b.End3 were left untreated (-) or stimulated with 50 ng/ml VEGF during 10 min (+). Samples were analyzed using Western blotting techniques. Proteins were visualized using phospho-specific antibodies against PRAS40-Thr246, PRAS40-Ser183, PKB/Akt-Ser473, PKB/Akt-Thr308, S6K-Thr389, S6K-Thr421/Tyr424, ERK1/2-Thr202/Tyr204 or total antibodies against PRAS40, PKB/Akt, S6K, ERK1/2*

Conclusion

Under conditions of insulin resistance, the insulin signalling hub PKB/Akt shows decreased activity. Many of PKB/Akt downstream phosphorylation substrates remain unknown. However, this thesis provides new information on PKB/Akt substrate PRAS40. PRAS40 is expressed in many different cell lines and cell types. With respect to signalling, PRAS40 is a component of both PI3K and mTORC1 mediated signalling. Different phosphorylation sites within the protein work together to ensure proper function of the protein. Due to the presence of a nuclear export signal in the protein, the ability of shuttling between the cytosolic and nuclear compartment is made possible. Furthermore, modification of the nuclear export signal or modification of expression of PRAS40 provides clear evidence that PRAS40 is an active player in the signalling processes controlling insulin resistance. Further research on PRAS40 could result in a better understanding of multiple disorders in which alterations in PI3K and mTORC1 mediated signalling are seen, such as in obesity, diabetes and certain cancers.

References

1. Taniguchi,CM, Emanuelli,B, Kahn,CR: Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85-96, 2006
2. McDonald,TJ, Nijland,MJ, Nathanielsz,PW: The insulin-like growth factor system and the fetal brain: effects of poor maternal nutrition. *Rev.Endocr.Metab Disord.* 8:71-84, 2007
3. Alessi,DR, Caudwell,FB, Andjelkovic,M, Hemmings,BA, Cohen,P: Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett.* 399:333-338, 1996
4. Obata,T, Yaffe,MB, Leparc,GG, Piro,ET, Maegawa,H, Kashiwagi,A, Kikkawa,R, Cantley,LC: Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J.Biol.Chem.* 275:36108-36115, 2000
5. Karlsson,HK, Zierath,JR, Kane,S, Krook,A, Lienhard,GE, Wallberg-Henriksson,H: Insulin-Stimulated Phosphorylation of the Akt Substrate AS160 Is Impaired in Skeletal Muscle of Type 2 Diabetic Subjects. *Diabetes* 54:1692-1697, 2005
6. Miinea,CP, Sano,H, Kane,S, Sano,E, Fukuda,M, Peranen,J, Lane,WS, Lienhard,GE: AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase activating protein domain. *Biochem.J.* 2005
7. Larance,M, Ramm,G, Stockli,J, van Dam,EM, Winata,S, Wasinger,V, Simpson,F, Graham,M, Junutula,JR, Guilhaus,M, James,DE: Characterisation of the role of the RabGAP AS160 in insulin-regulated GLUT4 trafficking. *J.Biol.Chem.* 2005
8. Eguez,L, Lee,A, Chavez,JA, Miinea,CP, Kane,S, Lienhard,GE, McGraw,TE: Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell Metab* 2:263-272, 2005
9. Ishikura,S, Bilan,PJ, Klip,A: Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. *Biochem.Biophys.Res.Commun.* 353:1074-1079, 2007
10. Sano,H, Eguez,L, Teruel,MN, Fukuda,M, Chuang,TD, Chavez,JA, Lienhard,GE, McGraw,TE: Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. *Cell Metab* 5:293-303, 2007
11. Cartee,GD, Wojtaszewski,JF: Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. *Appl.Physiol Nutr.Metab* 32:557-566, 2007
12. Burchfield,JG, Lennard,AJ, Narasimhan,S, Hughes,WE, Wasinger,VC, Corthals,GL, Okuda,T, Kondoh,H, Biden,TJ, Schmitz-Peiffer,C: Akt mediates insulin-stimulated phosphorylation of Ndrg2: evidence for cross-talk with protein kinase C theta. *J.Biol.Chem.* 279:18623-18632, 2004
13. Murray,JT, Campbell,DG, Morrice,N, Auld,GC, Shpiro,N, Marquez,R, Peggie,M, Bain,J, Bloomberg,GB, Grahammer,F, Lang,F, Wulff,P, Kuhl,D, Cohen,P: Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem.J.* 384:477-488, 2004
14. Yao,L, Zhang,J, Liu,X: NDRG2: a Myc-repressed gene involved in cancer and cell stress. *Acta Biochim.Biophys.Sin.(Shanghai)* 40:625-635, 2008
15. Kovacina,KS, Park,GY, Bae,SS, Guzzetta,AW, Schaefer,E, Birnbaum,MJ, Roth,RA: Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. *J.Biol.Chem.* 278:10189-10194, 2003
16. Madhunapantula,SV, Sharma,A, Robertson,GP: PRAS40 deregulates apoptosis in malignant melanoma. *Cancer Res.* 67:3626-3636, 2007
17. Zhang,F, Beharry,ZM, Harris,TE, Lilly,MB, Smith,CD, Mahajan,S, Kraft,AS: PIM1 protein kinase regulates PRAS40 phosphorylation and mTOR activity in FDCP1 cells. *Cancer Biol.Ther.* 8:846-853, 2009
18. Oshiro,N, Takahashi,R, Yoshino,KI, Tanimura,K, Nakashima,A, Eguchi,S, Miyamoto,T, Hara,K, Takehana,K, Avruch,J, Kikkawa,U, Yonezawa,K: The proline-Rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mTOR complex 1. *J.Biol Chem.* 282:20329-20339, 2007

19. Fonseca,BD, Smith,EM, Lee,VH, Mackintosh,C, Proud,CG: PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signalling downstream of this complex. *J.Biol Chem.* 282:24514-24524, 2007
20. Wang,L, Harris,TE, Lawrence,JC, Jr.: Regulation of proline-rich Akt substrate of 40 kDa (PRAS40) function by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation. *J.Biol Chem.* 283:15619-15627, 2008
21. Jazet,IM, Schaart,G, Gastaldelli,A, Ferrannini,E, Hesselink,MK, Schrauwen,P, Romijn,JA, Maassen,JA, Pijl,H, Ouwens,DM, Meinders,AE: Loss of 50% of excess weight using a very low energy diet improves insulin-stimulated glucose disposal and skeletal muscle insulin signalling in obese insulin-treated type 2 diabetic patients. *Diabetologia* 51:309-319, 2008
22. Saito,A, Narasimhan,P, Hayashi,T, Okuno,S, Ferrand-Drake,M, Chan,PH: Neuroprotective role of a proline-rich Akt substrate in apoptotic neuronal cell death after stroke: relationships with nerve growth factor. *J.Neurosci.* 24:1584-1593, 2004
23. Saito,A, Hayashi,T, Okuno,S, Nishi,T, Chan,PH: Modulation of proline-rich akt substrate survival signalling pathways by oxidative stress in mouse brains after transient focal cerebral ischemia. *Stroke* 37:513-517, 2006
24. Sgobbo,P, Pacelli,C, Grattagliano,I, Villani,G, Cocco,T: Carvedilol inhibits mitochondrial complex I and induces resistance to H2O2 -mediated oxidative insult in H9C2 myocardial cells. *Biochim.Biophys.Acta* 1767:222-232, 2007
25. Oprescu,AI, Bikopoulos,G, Naassan,A, Allister,EM, Tang,C, Park,E, Uchino,H, Lewis,GF, Fantus,IG, Rozakis-Adcock,M, Wheeler,MB, Giacca,A: Free Fatty Acid-Induced Reduction in Glucose Stimulated Insulin Secretion Evidence for a Role of Oxidative Stress In Vitro and In Vivo. *Diabetes* 2007
26. DeYoung,MP, Horak,P, Sofer,A, Sgroi,D, Ellisen,LW: Hypoxia regulates TSC1/2-mTOR signalling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes & Development* 22:239-251, 2008
27. Wang,L, Harris,TE, Roth,RA, Lawrence,JC: PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. *J.Biol Chem.* 2007
28. Roskoski,R, Jr.: Vascular endothelial growth factor (VEGF) signalling in tumor progression. *Crit Rev.Oncol.Hematol.* 62:179-213, 2007
29. Hennessy,BT, Smith,DL, Ram,PT, Lu,Y, Mills,GB: Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat.Rev.Drug Discov.* 4:988-1004, 2005
30. Shaw,RJ, Cantley,LC: Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* 441:424-430, 2006
31. Dunlop,EA, Tee,AR: Mammalian target of rapamycin complex 1: Signalling inputs, substrates and feedback mechanisms. *Cell Signal.* 2009
32. Shaw,RJ, Cantley,LC: Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature* 441:424-430, 2006
33. Rosner,M, Hanneder,M, Siegel,N, Valli,A, Fuchs,C, Hengstschlager,M: The mTOR pathway and its role in human genetic diseases. *Mutat.Res.* 659:284-292, 2008