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Beacon interacts with cdc2/cdc28-like kinases☆

Lakshmi Kantham,[a,*] Lyndal Kerr-Bayles,[a] Nathan Godde,[a] Melissa Quick,[a] Ryan Webb,[a] Terry Sunderland,[a] Judy Bond,[a] Ken Walder,[a] Guy Augert,[b] and Greg Collier[a,c]

[a] *Metabolic Research Unit, School of Health Sciences, Deakin University, Waurn Ponds 3217, Vic., Australia*
[b] *Merck Santé, CTGF, 885 Av Eugène Avinée, 59120 Loos, France*
[c] *Autogen Ltd., Pigdons Road, Waurn Ponds 3217, Vic., Australia*

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Abstract

Previously we found elevated beacon gene expression in the hypothalamus of obese *Psammomys obesus*. Beacon administration into the lateral ventricle of *P. obesus* stimulated food intake and body weight gain. In the current study we used yeast two-hybrid technology to screen for proteins in the human brain that interact with beacon. CLK4, an isoform of cdc2/cdc28-like kinase family of proteins, was identified as a strong interacting partner for beacon. Using active recombinant proteins and a surface plasmon resonance based detection technique, we demonstrated that the three members of this subfamily of kinases (CLK1, 2, and 4) all interact with beacon. Based on the known sequence and functional properties of beacon and CLKs, we speculate that beacon could either modulate the function of key regulatory molecules such as PTP1B or control the expression patterns of specific genes involved in the central regulation of energy metabolism.

Keywords: Beacon; CLK; cdc2/cdc28-like kinase; Obesity; Protein/protein interactions; SPR; Yeast two-hybrid; Ubiquitin; Sentrin

Psammomys obesus (commonly known as the Israeli Sand Rat) is a unique animal model for investigating the underlying molecular and signalling mechanisms that contribute to maintenance of energy balance [1,2]. Beacon was originally discovered using differential display PCR as a gene expressed at higher levels in the hypothalamus of obese *P. obesus* compared to their lean littermates. In further studies, ICV administration of beacon into lean *P. obesus* stimulated food intake and produced significant weight gain accompanied by a twofold increase in the hypothalamic gene expression of NPY [3]. Beacon treated animals revealed that the increase in body weight was a direct consequence of increased food intake as the treatment affected neither the physical activity nor the energy expenditure. The increase in body weight corresponded largely to an increase in fat content as the weight of the other organs remained unchanged [4].

The *P. obesus* beacon gene encodes a protein of 73 amino acids that is highly conserved across species and shares 100% sequence identity with the corresponding human and mouse homologs [3]. A number of peptides either produced or acting in the brain are involved in a complex network of neuronal signalling processes that control energy intake and energy expenditure. Beacon showed slight sequence homology to ubiquitins but not to any of the known peptides involved in the control of energy balance. Absence of a diglycine motif in the C-terminus precludes any typical ubiquitin like function for beacon [5]. As part of our efforts towards understanding how beacon functions at the molecular level, we investigated the nature of proteins it interacts with. Here we provide evidence to show that beacon interacts with three members of a distinct subfamily of kinases, termed cdc2/cdc28-like kinases abbreviated as CLKs [6].

☆ *Abbreviations:* CLK, cdc2/cdc28-like kinase; ICV, intracerebroventricular; NPY, neuropeptide Y; GST, glutathione *S*-transferase; IPTG, isopropyl thio-β-galactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; PTP1B, protein tyrosine phosphatase 1B; SR-proteins, serine/arginine-rich proteins; α-MSH, α-melanocyte stimulating hormone.

* Corresponding author. Fax: +61-3-5227-2170.
E-mail address: kantham@deakin.edu.au (L. Kantham).

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We discuss the significance of the interaction between beacon and CLKs in the context of known and speculated functions of CLKs in cell signalling and how this interaction may relate to central regulation of energy balance.

Methods

Yeast two-hybrid screen. Yeast two-hybrid screening with the ProQuest two-hybrid system (Life Technologies) was performed as described in [7]. The entire coding sequence of beacon (GenBank Accession No. AF318186) was cloned into the yeast vector pDBLeu, in fusion with the reading frame of GAL4 DNA binding domain. MaV203 transformed with pDBLeu-beacon were grown on plates containing 20 mM 3-amino-1,2,4-triazole (3AT) in order to suppress basal expression of *HIS3*. MaV203 cells harbouring pDBLeu-beacon were transformed with 18 μg of plasmid DNA harvested from a ProQuest human brain cDNA library and 1.4×10^6 transformants were screened for beacon interacting clones. Clones deemed HIS^+ positive in the primary screen were further screened for induction of two other test reporter genes, *URA3* and *lacZ*.

Expression and purification of recombinant GST and beacon proteins. The cDNA encoding beacon was subcloned into the pGEX2T expression vector (Amersham Pharmacia Biotech). Both GST and GST-beacon fusion protein were expressed in the BL21 strain of *Escherichia coli*. Cultures were grown at 37 °C and induced at 30 °C with 0.5 mM IPTG for 3 h. Bacteria were harvested and lysed by sonication, and the GST and GST-beacon fusion protein were affinity purified on Glutathione–Sepharose beads (Amersham Biosciences) using standard protocols. Protease inhibitor cocktail (Roche Molecular Biology) was added to the buffers during isolation. Over 25 mg of GST and GST-beacon was recovered per litre of culture. The GST tag was cleaved off using bovine plasma thrombin (Sigma) and further purified to homogeneity by removal of contaminating GST using Glutathione–Sepharose beads. Standard methods were used for SDS–PAGE and staining of gels by Coomassie blue to monitor the quantity and quality of proteins throughout the purification procedures.

Expression and purification of recombinant human CLK1, 2, and 4 proteins. Human liver cell line, HepG2, was used as a source for isolation of CLK clones. Human CLK1, 2, and 4 (GenBank Accession No. L29219, L29218, and AF294429) were amplified using the gene specific primers, forward 5′-gat tcc cgt gat tgc gtt aca-3′ and reverse 5′-gaa aaa gat gtt cat tac ctt agc-3′ for CLK1; and forward 5′-acg gac ttc ctg tgg gac aag c-3′ and reverse 5′-ctg gac tgg aca ccc act gct at-3′ for CLK2; forward 5′-agg agg gaa gac ggc agt ttg-3′ and reverse 5′-tag taa gac cac tga ttc cca ttt c-3′ for CLK4. Each insert was sequence verified and subcloned into the pGEX4T-1 expression vector (Amersham Biosciences). The GST-CLK proteins were expressed and purified as described above. However, the bacterial cultures were induced with IPTG at 25 °C for CLK1 and at 37 °C for CLK2 and CLK4. The GST-CLKs expressed in low amounts and on purification yielded 30, 65, and 700 μg CLK1, 2, and 4 fusion proteins per litre culture, respectively. Despite low yields, it was possible to concentrate and equilibrate the proteins into Biacore compatible 10 mM Hepes, pH 7.4 and 0.15 M NaCl buffer (HBS–N buffer from Biacore AB) using Centricon filters (Millipore) with 5 kDa molecular weight cutoff.

Kinase assay. Recombinant proteins were incubated in a kinase reaction buffer constituting 20 mM Tris–Cl, pH 7.4; 19 mM $MgCl_2$; 1 mM EGTA; and 0.018 μM ATP. Ten μCi [γ-^{32}P]ATP (New England Nuclear) was incorporated into 30 μl of reaction medium and incubated at room temperature for 20 min. Reactions were stopped by the addition of 15 μl of 5× Laemmli sample buffer. After performing SDS–PAGE, gels were exposed to a Phosphor Imager plate for 30 min and scanned using a Phosphor Imager (Molecular Dynamics).

Surface plasmon resonance analyses. Surface plasmon resonance (SPR) analyses were performed with minor variations as described in [7] using a Biacore J instrument (Biacore AB). Beacon was attached to a CM5 sensor chip at pH 5.5 by an amine coupling reaction. HBS–EP (10 mM Hepes, pH 7.4; 0.15 M NaCl; 3 mM EDTA; and 0.005% Polysorbate 20) was used as running buffer. Flow cells 1 and 2 in the sensor chip were treated in the same manner except that ligand was absent in flow cell 2 which served as a reference cell. Binding experiments were performed in dual channel mode and the reference subtracted sensorgrams represented true binding patterns between ligand and analyte. Test samples diluted to 130 μl in running buffer were injected over a fixed duration of 4 min. For heat treatments, proteins were initially diluted to 20 μl in running buffer, incubated at 65 or 70 °C for 10 min, centrifuged for 30 s, reconstituted to a final volume of 130 μl with running buffer, and injected as usual. Between injections, chips were regenerated using 40 μl of 10 mM NaOH. Sensorgrams were rejected if there was any problem in obtaining steady baseline prior to injections or if the chip regeneration was not satisfactory or if the chip showed any signs of deterioration.

Results

Using beacon as bait, we have screened over one million clones from a human brain cDNA library and identified 32 HIS^+ clones. Three of these (clones 12, 16, and 31) also induced expression of the other two reporter genes URA3 and lacZ. To determine the authenticity of the observed interactions, plasmids from the interacting clones were isolated, reintroduced into MaV203 cells containing the bait pDBLeu-beacon, and assayed for the expression of all three test reporter genes. The results were reproducible and confirmed positive (Fig. 1). When compared with the supplied

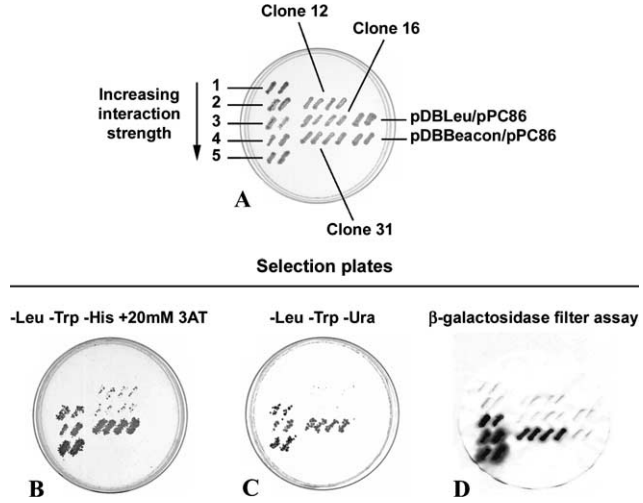

Fig. 1. Interaction between beacon, HSPB2, and CLK4-partial in the yeast two-hybrid assay. MaV203 cells were co-transformed with pDBBeacon and either clone 12 (HSPB2), clone 16 (HSPB2), or clone 31 (CLK4Partial), and patched onto selective media lacking leucine and tryptophan [-(Leu,Trp)] (A). Interactions were confirmed by growth on -(Leu, Trp, His) + 20 mM 3AT (B) and -(Leu, Trp, Ura) (C); and by monitoring the color development in a β-galactosidase filter assay (D). (B–D) Replicas of the master plate (A). Strength of the interaction is demonstrated by comparison to control strains 1–5.

positive controls, interactions between beacon and clones 12 and 16 were assessed to be weak and clone 31 to be a strong interaction (Fig. 1).

Sequencing revealed the two weakly interacting clones to be 100% homologous to human heat shock protein 2 (HSPB2, GenBank Accession No. NM_001541). The nucleotide sequence of clone 31, which interacted strongly with beacon, was revealed to be a partial clone and 100% homologous to the sequence of human cdc2/cdc28-like kinase 4 (CLK4; GenBank Accession No. AF294429). The cDNA corresponding to the 249 bp beginning from the start codon of CLK4 was absent in clone 31, indicating that the fusion protein expressed was incomplete, lacking the first 83 amino acids of CLK4.

Human CLK4 shares 68%, 67%, and 63% sequence identity at the amino acid level with its three closely related family members CLK1, 2, and 3, respectively. CLK1, 2, and 4 were shown to be expressed in the brain and several other tissues [6,8]. CLK1 and 2 have the ability to phosphorylate and potentiate the activity of PTP1B [9]. PTP1B is one of the most prominent non-transmembrane, cytosolic phosphatases implicated in the regulation of a variety of receptor mediated intracellular signalling pathways including those of insulin and leptin [10,11]. To further confirm the interaction between beacon and CLK4 and to examine whether beacon similarly interacts with CLK1 and 2, we employed SPR, a highly sensitive in vitro technique for monitoring protein/protein interactions in real time.

Beacon, GST, GST-CLK1, GST-CLK2, and GST-CLK4 were expressed and purified as recombinant proteins. The GST and beacon produced were pure and homogeneous. Conversely, the CLKs expressed poorly, but were amenable to purification and tended to break down giving rise to protein fragments of varying lengths. All three CLKs exhibited kinase activity (Fig. 2),

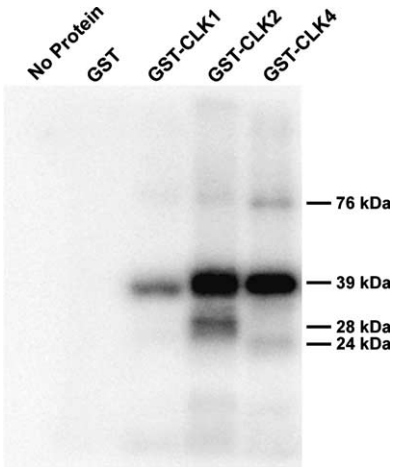

Fig. 2. Kinase activity of CLK proteins kinase assay was carried out as described under Methods. No protein (No protein); GST (19 μg); GST-CLK1 (3 μg); GST-CLK2 (6 μg); and GST-CLK4 (19 μg).

indicating the proteins to be authentic and functional. Beacon was not phosphorylated by any of the CLKs and therefore is unlikely to be one of the protein substrates for CLKs.

In SPR analyses each GST-CLK fusion protein showed concentration dependent binding to beacon (Figs. 3A–C). GST alone did not show any binding to beacon which suggests that the GST component of the GST-CLKs was not responsible for the observed binding phenomenon (data not shown). The activity of CLKs responsible for binding to beacon appeared to be unstable to treatments at higher temperatures. The binding of beacon to CLK1 and 2 decreased by >90% when treated at 65 °C for 10 min (Fig. 3D,E). The binding activity of CLK4 decreased by 60% when treated at 70 °C (Fig. 3F). Further, beacon or other unrelated proteins such as bovine serum albumin, maltose binding protein or insulin did not show binding to beacon (data not shown), suggesting the beacon/CLK interactions to be highly specific.

Discussion

In the yeast two-hybrid screen, we identified human CLK4 to be a potential interacting partner for beacon. Subsequently, we demonstrated that three members of the human CLK family (CLK1, 2, and 4) interact with beacon and that this interaction is highly specific. The CLKs belong to the "LAMMER" family of kinases, named for the presence of "EHLAMMERILG" signature motif in the substrate binding cleft. They share high sequence homology, occur in a range of species from yeast to humans, and are characterized by the presence of a highly conserved catalytic C-terminal domain and a less conserved serine/arginine rich N-terminal regulatory domain. They are dual specific kinases and have the ability to *cis* and *trans* phosphorylate serine, threonine, and tyrosine residues on target proteins [12]. Occurrence of alternative splice variants and truncated forms, as well as their expression in a tissue specific manner, appears to be a common feature of CLKs [13]. The CLK proteins are located in the nucleus as well as in the cytoplasm. A consensus sequence motif for phosphorylation by CLKs is present in PTP1B, a negative regulator of both insulin and leptin signalling [14]. Co-expression and in vitro phosphorylation studies indicate that CLK1 and 2 may phosphorylate and activate PTP1B in vivo [9]. PTP1B knock-out mice were found to be lean with increased sensitivity to insulin and leptin, providing compelling evidence for its effects on the pathways controlling energy homeostasis [15,16]. PTP1B is currently a popular target for development of anti-obesity and diabetes drugs [17]. Our findings provide new impetus to explore the possibility whether beacon, CLKs, PTP1B, insulin, and leptin receptors are linked in a

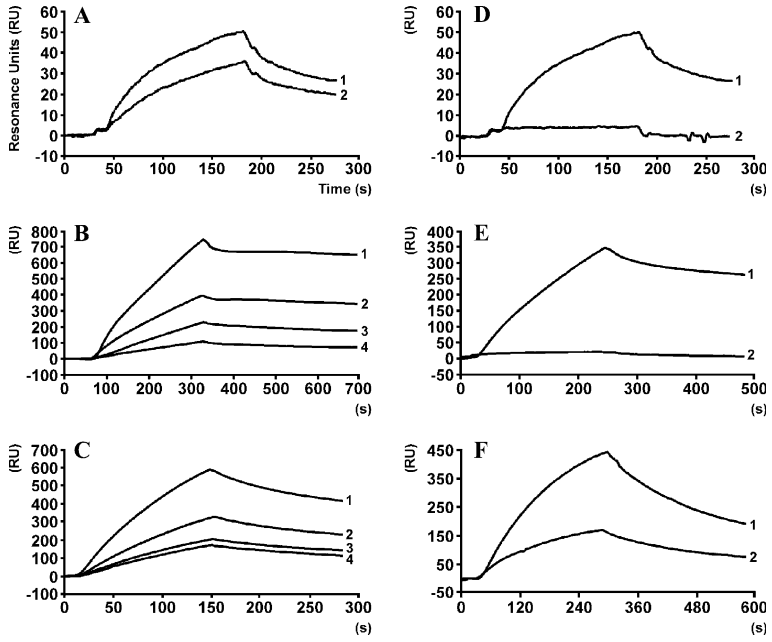

Fig. 3. Interaction between beacon and human CLK1, 2, and 4. SPR analyses were performed as described in Methods. Beacon immobilized on CM5 sensor chip generated 1800–2100 RU. Analyte samples injected and the heat treatments are as listed here. (A) GST-CLK1 (1: 1.68 μg; 2: 0.84 μg). (B) GST-CLK2 (1: 1.56 μg; 2: 0.78 μg; 3: 0.50 μg; and 4: 0.26 μg). (C) GST-CLK4 (1: 0.98 μg; 2: 0.49 μg; 3: 0.33 μg; and 4: 0.24 μg). (D) GST-CLK1 (1: 1.68 μg; 2: 1.68 μg treated at 65 °C for 10 min). (E) GST-CLK2 (1: 1.00 μg; 2: 1.00 μg treated at 65 °C for 10 min). (F) GST-CLK4 (1: 1.30 μg; 2: 1.30 μg treated at 70 °C for 10 min).

pathway that is active in vivo and whether such a pathway has any bearing on the control of energy metabolism.

The CLKs, via their kinase activity, may regulate the sub-nuclear distribution of SR-proteins, accessory factors involved in pre-mRNA splicing. Cellular regulation of kinase activity of CLKs is modular, highly complicated and involves an inter play between several kinases and phosphatases. It was shown that the CLKs regulate splicing of their own mRNA, resulting in the formation of catalytically active or inactive variants. Active CLKs phosphorylate SR-proteins and the activated SR-proteins in turn control the exon exclusion and formation of catalytically inactive forms of CLKs [18,19]. Dysregulated activity of CLK isoforms or an incorrect distribution of CLKs between the cytoplasm and nucleus could have serious implications in the splicing and expression pattern of numerous genes. However, the subtle mechanisms that underlie intracellular trafficking and regulation of the kinase activity of CLKs are not understood.

It was found that beacon binds to CLKs but it is not a substrate for phosphorylation. Close examination of sequences revealed the presence of a potential sentrinization sequence motif "LKPE" in CLK1, 2 and 4. In addition, CLK4 has a second sequence motif "VKSD" also considered a potential site for sentrinization [20]. Beacon differs from ubiquitins and sentrins by the presence of a dityrosine motif in its C-terminus in the place of a diglycine in the latter. One interesting possibility might be that beacon is a novel form of ubiquitin with potential to target and modify specific proteins via the dityrosine motif using novel processing and conjugation systems [21]. Indirect evidence exists for such a possibility in yeast [22] but is hitherto unknown in higher eukaryotes. Our knowledge of regulatory functions mediated by ubiquitins, ubiquitin like or ubiquitin domain containing proteins is rapidly growing and it is conceivable that the extent of regulation of cellular pathways controlled by the ubiquitin family of proteins may far exceed that of phosphorylation–dephosphorylation mediated control mechanisms [23–27]. The mouse coat color mutant gene "mahoganoid" has been implicated to have modulatory effects on α-MSH signalling pathway in the brain and thereby influencing the body weight in mice. Mahoganoid has recently been cloned and identified as a ring domain protein and may function as an E3 ubiquitin ligase [28]. It would be interesting to know the type of ubiquitins and the target proteins that may be involved in the ubiquitination processes mediated by mahoganoid. It remains to be addressed whether the interaction we observed in vitro relates to sequestering of CLKs or a step in the process of covalent modification by beacon in vivo and whether such modification can influence their subcellular localization or level of kinase activity. Either of these processes could influence one or more signalling pathways modulated by CLKs or substrates of CLKs.

Our findings shed new light on the functional aspects of beacon and the CLK family of proteins. Further work is needed to resolve whether beacon via its interaction with CLKs modulates PTP1B activity or controls the expression of particular but as yet undefined genes in the brain that are involved in the maintenance of energy balance.

References

[1] K.R. Walder, R.P. Fahey, G.J. Morton, P.Z. Zimmet, G.R. Collier, Characterization of obesity phenotypes in *Psammomys obesus* (Israeli sand rats), Int. J. Exp Diabetes Res. 1 (2000) 177–184.

[2] K. Walder, N. Oakes, R.P. Fahey, G. Cooney, P.Z. Zimmet, G.R. Collier, Profile of dyslipidemia in *Psammomys obesus*, an animal model of the metabolic syndrome, Endocr. Regul. 36 (2002) 1–8.

[3] G.R. Collier, J.S. McMillan, K. Windmill, K. Walder, J. Tenne-Brown, A. de Silva, J. Trevaskis, S. Jones, G.J. Morton, S. Lee, G. Augert, A. Civitarese, P.Z. Zimmet, Beacon: a novel gene involved in the regulation of energy balance, Diabetes 49 (2000) 1766–1771.

[4] K. Walder, J.S. McMillan, S. Lee, A. Civitarese, P. Zimmet, G.R. Collier, Effects of beacon administration on energy expenditure and substrate utilisation in *Psammomys obesus* (Israeli sand rats), Int. J. Obes. Relat. Metab. Disord. 25 (2001) 1281–1285.

[5] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev. Biochem. 67 (1998) 425–479.

[6] O. Nayler, S. Stamm, A. Ullrich, Characterization and comparison of four serine- and arginine-rich (SR) protein kinases, Biochem. J. 326 (Pt 3) (1997) 693–700.

[7] K. Walder, L. Kantham, J.S. McMillan, J. Trevaskis, L. Kerr, A. De Silva, T. Sunderland, N. Godde, Y. Gao, N. Bishara, K. Windmill, J. Tenne-Brown, G. Augert, P.Z. Zimmet, G.R. Collier, Tanis: a link between type 2 diabetes and inflammation? Diabetes 51 (2002) 1859–1866.

[8] J. Schultz, T. Jones, P. Bork, D. Sheer, S. Blencke, S. Steyrer, U. Wellbrock, D. Bevec, A. Ullrich, C. Wallasch, Molecular characterization of a cDNA encoding functional human CLK4 kinase and localization to chromosome 5q35 [correction of 4q35], Genomics 71 (2001) 368–370.

[9] F.M. Moeslein, M.P. Myers, G.E. Landreth, The CLK family kinases, CLK1 and CLK2, phosphorylate and activate the tyrosine phosphatase, PTP-1B, J. Biol. Chem. 274 (1999) 26697–26704.

[10] J.C. Byon, A.B. Kusari, J. Kusari, Protein-tyrosine phosphatase-1B acts as a negative regulator of insulin signal transduction, Mol. Cell Biochem. 182 (1998) 101–108.

[11] J.M. Zabolotny, K.K. Bence-Hanulec, A. Stricker-Krongrad, F. Haj, Y. Wang, Y. Minokoshi, Y.B. Kim, J.K. Elmquist, L.A. Tartaglia, B.B. Kahn, B.G. Neel, PTP1B regulates leptin signal transduction in vivo, Dev. Cell 2 (2002) 489–495.

[12] K. Lee, C. Du, M. Horn, L. Rabinow, Activity and autophosphorylation of LAMMER protein kinases, J. Biol. Chem. 271 (1996) 27299–27303.

[13] P.I. Duncan, B.W. Howell, R.M. Marius, S. Drmanic, E.M. Douville, J.C. Bell, Alternative splicing of STY, a nuclear dual specificity kinase, J. Biol. Chem. 270 (1995) 21524–21531.

[14] A. Cheng, N. Uetani, P.D. Simoncic, V.P. Chaubey, A. Lee-Loy, C.J. McGlade, B.P. Kennedy, M.L. Tremblay, Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B, Dev. Cell 2 (2002) 497–503.

[15] M. Elchebly, P. Payette, E. Michaliszyn, W. Cromlish, S. Collins, A.L. Loy, D. Normandin, A. Cheng, J. Himms-Hagen, C.C. Chan, C. Ramachandran, M.J. Gresser, M.L. Tremblay, B.P. Kennedy, Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene, Science 283 (1999) 1544–1548.

[16] L.D. Klaman, O. Boss, O.D. Peroni, J.K. Kim, J.L. Martino, J.M. Zabolotny, N. Moghal, M. Lubkin, Y.B. Kim, A.H. Sharpe, A. Stricker-Krongrad, G.I. Shulman, B.G. Neel, B.B. Kahn, Increased energy expenditure, decreased adiposity, and tissue-specific insulin sensitivity in protein-tyrosine phosphatase 1B-deficient mice, Mol. Cell Biol. 20 (2000) 5479–5489.

[17] O. Ukkola, M. Santaniemi, Protein tyrosine phosphatase 1B: a new target for the treatment of obesity and associated co-morbidities, J. Int. Med. 251 (2002) 467–475.

[18] P.I. Duncan, D.F. Stojdl, R.M. Marius, J.C. Bell, In vivo regulation of alternative pre-mRNA splicing by the Clk1 protein kinase, Mol. Cell Biol. 17 (1997) 5996–6001.

[19] R. Katsu, H. Onogi, K. Wada, Y. Kawaguchi, M. Hagiwara, Novel SR-rich-related protein clasp specifically interacts with inactivated Clk4 and induces the exon EB inclusion of Clk, J. Biol. Chem. 277 (2002) 44220–44228.

[20] E.T. Yeh, L. Gong, T. Kamitani, Ubiquitin-like proteins: new wines in new bottles, Gene 248 (2000) 1–14.

[21] K.I. Kim, S.H. Baek, C.H. Chung, Versatile protein tag, SUMO: its enzymology and biological function, J. Cell Physiol. 191 (2002) 257–268.

[22] G.A. Dittmar, C.R. Wilkinson, P.T. Jedrzejewski, D. Finley, Role of a ubiquitin-like modification in polarized morphogenesis, Science 295 (2002) 2442–2446.

[23] A.N. Hegde, A. DiAntonio, Ubiquitin and the synapse, Nat. Rev. Neurosci. 3 (2002) 854–861.

[24] C.M. Pickart, Ubiquitin enters the new millennium, Mol. Cell 8 (2001) 499–504.

[25] M. Hochstrasser, Evolution and function of ubiquitin-like protein-conjugation systems, Nat. Cell Biol. 2 (2000) E153–E157.

[26] S. Jentsch, G. Pyrowolakis, Ubiquitin and its kin: how close are the family ties? Trends Cell Biol. 10 (2000) 335–342.

[27] D.M. Koepp, J.W. Harper, S.J. Elledge, How the cyclin became a cyclin: regulated proteolysis in the cell cycle, Cell 97 (1999) 431–434.

[28] L.K. Phan, F. Lin, C.A. LeDuc, W.K. Chung, R.L. Leibel, The mouse mahoganoid coat color mutation disrupts a novel C3HC4 RING domain protein, J. Clin. Invest. 110 (2002) 1449–1459.