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Regulation of protein phosphatases in disease and behaviour

Meeting on protein phosphatases

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The EMBO conference/FEBS advanced course on phosphatases, 'EuroPhosphatases 2003', was held in Barcelona, Spain, between 29 June and 3 July 2003, and was organized by J. Arino and D. Alexander.

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Introduction

The biennial EuroPhosphatases meeting was held near the Sagrada Família, Antonio Gaudi's unfinished architectural masterpiece, which could be a metaphor for the phosphatase field: a lot has been achieved, but much more work still needs to be done. Protein phosphorylation is reversible and has a regulatory role in many—if not all—biological processes. The enzymes involved in protein dephosphorylation, the protein phosphatases (PPs), are classified on the basis of their substrate specificity into serine/threonine PPs, protein-tyrosine phosphatases (PTPs) and protein-histidine phosphatases (PHPs). Molecular cloning has indicated that these three classes belong to four gene families, two of which, the PPP and the PPM family, encode the Ser/Thr PPs (Barford, 1996). The Ser/Thr PPs consist of a single catalytic subunit and one or more regulatory subunits. The large family of PTPs consists of classical PTPs with substrate specificity for phosphotyrosine (pTyr), dual-specificity phosphatases (DSPs) that dephosphorylate pSer, pThr and pTyr, and lipid phosphatases (Tonks & Neel, 2001). Finally, little is known about histidine phosphorylation in vertebrates. S. Klumpp (Munster, Germany) described the purification and cloning of the only known mammalian PHP, PHP1, which is not homologous to any other PP (Klumpp & Krieglstein, 2002).

The general view of dynamic cell signalling through phosphorylation is changing. D. Brautigan (Charlottesville, VA, USA) argued that PP activity should no longer be considered to be at a constant, low level, but rather that a relatively high level of PP activity keeps the cell in check until a stimulus tips the protein kinase (PK)/PP balance through the simultaneous activation of PKs and the inactivation of PPs (Fig. 1). This model implies that PPs are tightly regulated. This regulation, together with the elucidation of the *in vivo* function of PPs, were important themes of the meeting.

Regulation of protein phosphatases

Redox signalling. The catalytic sites of PTPs contain a conserved cysteine that is directly involved in catalysis. Catalytic cysteines have a low pK_a because of their micro-environment, and they are susceptible to oxidation. Many stimuli induce the production of reactive oxygen species, such as the second messenger H_2O_2, and these cause the reversible conversion of the cysteine thiolate anion to sulphenic acid and the resultant inactivation of PTP activity. On further oxidation, this is irreversibly converted to sulphinic and sulphonic acid (Fig. 2A). D. Barford (London, UK) presented the crystal structure of oxidized PTP1B, which revealed an unusual five-membered ring, cyclic sulphenamide, with a covalent bond between the sulphur of Cys 215 and the backbone nitrogen of Ser 216. The formation of cyclic sulphenamide was rapid, as no sulphenic acid forms were detected on oxidation. Cyclic sulphenamide cannot be further oxidized to sulphinic and sulphonic acid and so protects PTP1B against irreversible inactivation, but it is reduced by thiols, including

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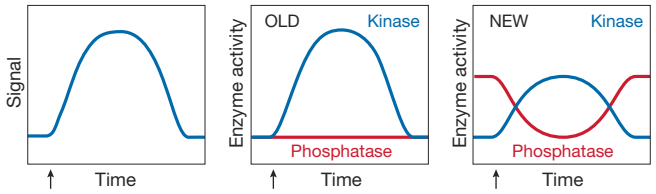

Fig. 1 | Phosphorylation-mediated signalling. A stimulus (arrow) induces a transient rise in protein phosphorylation for signalling (left panel). This was thought to be due solely to an increase in protein kinase (PK) activity (OLD view, middle panel), whereas the NEW view is that the transient response is due to an increase in PK activity and a concomitant reduction in protein phosphatase activity (right panel). Adapted from a figure by D.L. Brautigan (Charlottesville, Virginia, USA).

glutathione. The oxidation of PTP1B induces major changes in the loops that form the catalytic site of PTP1B (Fig. 2B). Given the high sequence conservation in the catalytic sites of PTPs, it is anticipated that the oxidation of other PTPs will also lead to the formation of cyclic sulphenamide.

Receptor PTPs (RPTPs) generally contain two homologous PTP domains of which the membrane-proximal domain, D1, is catalytically active, whereas the membrane-distal domain, D2, is not. In addition, some RPTPs, including CD45 and RPTP-α, are regulated by dimerization. J. den Hertog (Utrecht, the Netherlands) provided evidence that RPTPs are inactivated by oxidation in an unexpected way. Rather than affecting the active D1 domain, oxidative stress induces a conformational change in D2 of the proto-typical RPTP-α, which leads to the stabilization of the dimeric form due to a change in the relative orientation of the two monomers in the dimer (rotational coupling). This results in a conformational change in the ectodomain and the inactivation of RPTP-α. All of these effects are dependent on the catalytic cysteine in RPTP-α-D2. In agreement with these findings, RPTP-α-D2, but not RPTP-α-D1, is oxidized in response to redox signalling, as assessed using an antibody generated by A. Ostman (Uppsala, Sweden) that allows the detection of oxidized catalytic cysteines in PTPs.

Oxidation is an appealing regulatory mechanism for PTPs. However, does oxidation of PTPs occur in response to physiological stimuli? N. Tonks (Cold Spring Harbor, NY, USA) addressed this question by using an SDS–PAGE in-gel phosphatase assay that was modified so that oxidized PTPs could be visualized. Insulin treatment leads to the oxidation of two PTPs, the highly homologous 45-kDa T-cell PTP (TC45) and PTP1B. The insulin receptor (IR) is a direct substrate of TC45 and PTP1B. Tonks showed that a substrate-trapping mutant of TC45 binds selectively to pTyr 972 of IR-β, and that a doubly phosphorylated peptide from IR-β containing pTyr 1162 and pTyr 1163 binds perfectly to PTP1B in co-crystals. He concluded that insulin signalling induces the transient oxidation and inactivation of two homologous PTPs that dephosphorylate distinct sites in the activated IR.

Indeed, redox signalling is emerging as an important regulatory mechanism for PTPs, which leads to the rapid, transient, stimulus-induced inactivation of PTPs that—given the high k_{cat} of PTPs—may be essential for tyrosine phosphorylation to occur in cells.

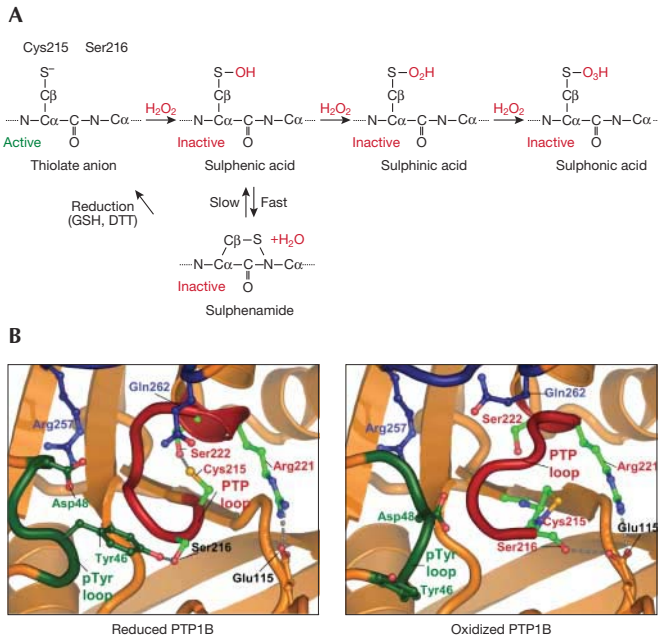

Fig. 2 | Oxidation of the catalytic Cys 215 in PTP1B. (**A**) Sulphenamide formation is reversible and protects against irreversible sulphinic and sulphonic acid formation. (**B**) Crystal structure of reduced (left) and oxidized (right) protein-tyrosine phosphatase (PTP)1B. On oxidation, cyclic sulphenamide forms, the PTP loop (red) opens up, and the pTyr loop (green) and Q-loop (blue) fold outwards, disallowing substrate binding and dephosphorylation at the same time (courtesy of D. Barford, London, UK).

Protein–protein interactions and phosphorylation

During the past decade, evidence has accumulated that Ser/Thr PPs are regulated by protein–protein interactions. Many proteins have been identified that bind to the catalytic subunit of Ser/Thr PPs, thereby regulating the catalytic activity or the subcellular localization of the PP. At the same time, the PP often regulates the function of the bound protein by dephosphorylation. M. Cyert (Stanford, CA, USA) found that the transcription factor Crz1 binds to the yeast Ser/Thr PP calcineurin (CN), which is activated in response to extracellular stimuli that raise intracellular free Ca^{2+} concentrations. This leads to the dephosphorylation of Crz1, its subsequent nuclear translocation and transcriptional activation. Using DNA microarrays, Cyert identified 163 genes that are upregulated in response to Ca^{2+} or Na^{+}, and are down-regulated by more than 50% in response to CN inhibition. In addition, all 119 yeast kinases were screened for their ability to phosphorylate Crz1. Hrr25, a yeast casein kinase Iδ/ε homologue, binds and phosphorylates Crz1 *in vivo*, and Hrr25 overexpression decreases Crz1-dependent transcription by 60%, which is probably due to the relocalization of Crz1 to the cytoplasm. Whether there is a trimeric complex consisting of Crz1, CN and Hrr25 remains to be determined.

PP2A is a heterotrimeric Ser/Thr PP that consists of a structural A subunit, a catalytic C subunit and a regulatory B subunit. The B subunit determines the catalytic activity, substrate specificity and subcellular localization of the holoenzyme, and four distinct

families of B subunit have been identified (B, B′, B″ and B‴). J. Goris (Leuven, Belgium) analysed the function of PR72, a regulatory subunit of the B″ family. PR72 contains two EF-hand motifs that bind Ca^{2+} differentially. EF2 determines Ca^{2+}-dependent binding of the B to the A subunit and the nuclear localization of the holoenzyme, whereas EF1 is, at least in part, required for the PR72-overexpression-induced G1/S block in the cell cycle. Taken together, the two EF-hand motifs in PR72 have distinct roles in the function of the PP2A holoenzyme.

The *Drosophila* genome encodes one A subunit, one C subunit and four B subunits (R2/B, R3/PR72, R5/B56-1 and its isoform R5/B56-2), which generates only four possible PP2A holoenzymes. M. Mumby (Dallas, TX, USA) used RNA interference in *Drosophila* Schneider 2 (S2) cells to knock down specific subunits of PP2A and thus study PP2A function. Only the PP2A holoenzyme is stable in living cells, as the knockdown of either the A or the C subunit leads to a reduction of all PP2A subunits. By using selective knockdown of the B subunits, Mumby showed that they differentially regulate PP2A function as the holoenzyme containing R2/B negatively regulates mitogen-activated protein kinase (MAPK) signalling, whereas those containing R5/B56-1 or -2 have anti-apoptotic activity.

Another regulator of PP2A is the α4 protein. S. Inui (Kumamoto, Japan) generated a mouse model known as lck-$\alpha4^{-/-}$ mice in which α4 is deleted in a tissue-specific manner in T cells. T-cell development is arrested at the $CD4^-/CD8^-$ double-negative stage and few mature thymocytes are produced because of impaired proliferation, rather than increased apoptosis. It will be interesting to unravel how the α4-mediated activation of PP2A contributes to thymocyte proliferation.

More than 70 proteins are known to interact with PP1, another Ser/Thr PP. M. Bollen (Leuven, Belgium) showed that the highly selective nuclear inhibitor of PP1 (NIPP1) localizes to subnuclear speckles and spliceosomes and binds not only to PP1, but also to two splicing factors, SAP155 and CDC5L, and MEL kinase, a kinase involved in splicing. These interactions are mediated by a forkhead-associated (FHA) domain in NIPP1. The MEL kinase-mediated phosphorylation of NIPP1 Thr 61, in the FHA domain, or of NIPP1 Ser 199 in the PP1-binding site, abolishes its interaction with ligands as well as its localization to nuclear speckles. The inactivation of NIPP1 by gene targeting in the mouse leads to embryonic lethality at day 6.5. Interestingly, NIPP1 binds to the embryonic ectoderm development (EED) protein, which is a polycomb-group protein that is implicated in the maintenance of homeotic genes in their repressed state, once they are inactivated during early development. Whether PP1 regulates EED function in the PP1–NIPP1–EED complex remains to be determined. NIPP1, initially identified as a PP1 inhibitor, therefore appears to be an adaptor protein that is involved in diverse signalling pathways.

Some protein inhibitors of PP1, such as I-1 and DARPP32, are activated by PKA. CPI-17 is the founding member of a separate family of PKC-activated inhibitors. Brautigan showed that phosphorylation of CPI-17 enhanced its PP1 inhibitory activity by 1,000-fold. CPI-17 phosphorylation occurs in intact smooth muscle cells in response to receptor activation. PP1 forms part of a larger holoenzyme complex and, interestingly, different protein inhibitors are specific for different PP1 holoenzymes. For example, pThr 38–CPI-17 inhibits PP1 in the myosin light chain phosphatase (MLCP) holoenzyme, but not PP1 bound to glycogen-targeting subunits, which indicates that the active site of these two holoenzymes might be different. PhosphoCPI-17 is rapidly dephosphorylated by glycogen-bound PP1, but not by MLCP, and this 'arrested hydrolysis' prolongs the inhibition of MLCP. Unlike NIPP1, CPI-17 is a bona fide PP1 inhibitor that has no recognized activity, other than PP1 inhibition.

Hal3, a flavoprotein, inhibits the yeast PP1-like Ppz1, a regulator of the cell cycle, by binding to its carboxy-terminal catalytic domain. Overexpression of Hal3 rescues the G1/S cell-cycle arrest of yeast cells that lack the PP2A-like Sit4. Therefore, Sit4 and Ppz1 have opposing roles in the G1/S cell-cycle transition. J. Arino (Barcelona, Spain) reported that the yeast genome encodes two additional Hal-like proteins, Vhs3 and YKL088w. Vhs3 inhibits Ppz1 activity and mimics Hal3 function, although less potently. YKL088w does not bind Ppz1 *in vitro* and overexpression of YKL088w leads to an anti-Hal3 phenotype, suggesting that YKL088w is a naturally occurring dominant-negative Hal3.

PPs may be regulated not only by protein–protein interactions, but also by direct phosphorylation. PTEN is an important tumour suppressor that belongs to the PTP superfamily, but which has lipid phosphatase activity that is specific for phosphatidylinositol-3-phosphate (PtdIns(3)P). R. Pulido (Valencia, Spain) identified many overlapping casein kinase 2 (CK2) phosphorylation sites in the C terminus of PTEN. This region encodes consensus caspase cleavage sites and PTEN is cleaved specifically by caspase 3. Interestingly, phosphorylation of PTEN *in vitro* inhibits caspase-3-mediated cleavage, and activation of caspase 3 leads to reduced PTEN protein levels in cells. This indicates that CK2-mediated phosphorylation regulates PTEN by inhibiting caspase-3-mediated cleavage.

In summary, PPs are tightly regulated by diverse mechanisms, including direct oxidation and phosphorylation, proteolysis, as well as by binding of inhibitor proteins that may, in turn, be regulated by phosphorylation or dephosphorylation.

In vivo functions of phosphatases

Protein phosphatases and disease. Because protein dephosphorylation is a key process involved in cell signalling, it is not surprising that PPs are implicated in diseases such as cancer and diabetes. Insulin signalling has an important role in obesity and obesity-induced type 2 diabetes. M. Tremblay (Montreal, Canada) showed that $PTP1B^{-/-}$ mice are resistant to diet-induced obesity. Distinct pathways are involved, including insulin signalling through the IR, and leptin and growth hormone signalling mediated by the receptor-associated protein tyrosine kinase, Jak2. The IR and Jak2 are direct substrates of PTP1B. PTP1B inhibitors are therefore being investigated as a treatment for obesity, but one concern is that they might also contribute to the development of cancer. The insulin-like growth factor 1 receptor (IGF1R) is highly homologous to the IR and it is hyperphosphorylated in $PTP1B^{-/-}$ cells, which leads to enhanced Akt phosphorylation and cell survival in response to serum deprivation and genotoxic stress. Nevertheless, $PTP1B^{-/-}$ mice do not go on to develop tumours because Ras signalling, which is required for tumorigenesis and maintenance, is impaired in the absence of PTP1B (Fig. 3). Activated V12Ras bypasses the inhibition caused by the loss of PTP1B (Fig. 3) and increases the transformation frequency of $PTP1B^{-/-}$ cells compared with wild-type cells. In $PTP1B^{-/-}$ mice lacking the tumour

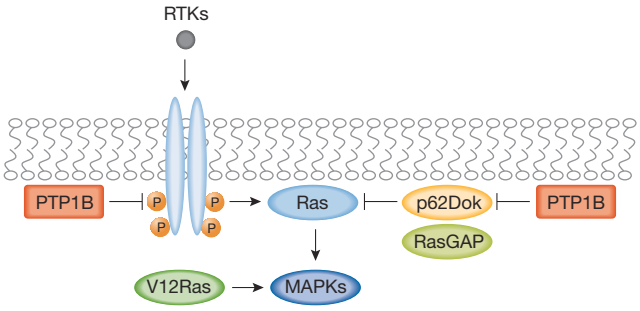

Fig. 3 | Impaired Ras signalling in PTP1B$^{-/-}$ cells. Loss of protein-tyrosine phosphatase (PTP)1B leads to increased receptor tyrosine kinase (RTK) phosphorylation and enhanced signalling of most downstream pathways. However, loss of PTP1B also leads to increased expression of RasGAP and to hyperphosphorylation of p62Dok, both leading to attenuation of Ras/mitogen-activated protein kinase (MAPK) signalling (courtesy of M. Tremblay, Montreal, Canada).

suppressor p53, tumour formation is enhanced, but no difference is found between PTP1B$^{+/-}$p53$^{-/-}$ and PTP1B$^{+/+}$p53$^{-/-}$ mice. Thus, half the normal level of PTP1B is enough to provide tumour protection and is sufficient for the treatment of diabetes type 2 and obesity, and so this is encouraging for the use of PTP1B inhibitors in patients.

Shp-2, a cytosolic PTP with two SH2 domains amino-terminal to its PTP domain, might be involved in cancer, because Shp-2 mutations have been found in 20–25% of juvenile myelomonocytic leukaemia (JMML) patients. Noonan syndrome (NS) is also linked to mutations in Shp-2. NS is a relatively common dominant autosomal disorder (incidence 1:1,000/1:2,500) that is characterized by dysmorphic facial features, proportionate short stature, heart disease and, importantly, an increased incidence of leukaemia. Shp-2 is negatively regulated by intramolecular binding of the PTP domain to the most N-terminal SH2 domain (N-SH2), which is alleviated when Shp-2 binds to doubly phosphorylated proteins. Most mutations in Shp-2 from NS and JMML patients are localized to the interface between the N-SH2 domain and the PTP domain. B. Neel (Boston, MA, USA) showed that mutant Shp-2 from NS and JMML patients has increased PTP activity, with the JMML Shp-2 being the most active, which indicates that these are gain-of-function mutations. Shp-2$^{-/-}$ cells show delayed spreading and defective migration, as well as an increased number of focal adhesions and stress fibres, suggesting that Shp-2 regulates one or more Src family kinases (SFKs) that are involved in these processes. Indeed, Shp2$^{-/-}$ cells show defective integrin-induced Src activation that might be due to the hyperphosphorylation of PAG, a Shp-2 binding protein and substrate that recruits C-terminal Src kinase (Csk), which leads to the hyperphosphorylation of Src pTyr 527 and thus to the inactivation of SFKs. Whether the effects of mutant Shp-2 from NS and JMML patients are mediated by SFK activation remains to be determined.

Shp-2 not only regulates SFKs, but also has a role in other PK cascades. Overexpression studies with wild-type and dominant-negative Shp-2 in IL-3-dependent haematopoietic cells show that Shp-2 has a positive role in extracellular signal-regulated kinase (ERK) activation and a negative role in JNK activation, and both of these effects lead to enhanced cell survival (M. Welham, Bath, UK). Shp-2 might also function as a scaffolding protein in lipopolysaccharide (LPS) signalling, as K. Forbes (Glasgow, UK) showed that both dominant-negative Shp-2 and wild-type Shp-2 enhance LPS-induced NF-κB transactivation, possibly through its association with I-κB kinase.

CD45 is a haematopoietic RPTP with apparent anti-tumorigenic activity in certain contexts. D. Alexander (Cambridge, UK) showed that the activating autophosphorylation site Tyr 394 in the SFK Lck, and the inhibitory Tyr 505, are hyperphosphorylated in CD45$^{-/-}$ cells. Therefore, CD45 has both positive and negative effects on Lck activity. The mutated Lck Y505F is hyperactive and highly tumorigenic when expressed in CD45$^{-/-}$ cells, but not in CD45$^{+/+}$ cells. The comparison of pre-tumorigenic CD45$^{-/-}$/Lck Y505F with CD45$^{+/+}$/Lck Y505F thymocytes allowed Alexander to dissect the mechanisms that underlie tumorigenesis. There are no differences in cell survival or the cell cycle, but hyperactive Lck Y505F inhibits DNA repair, promotes genomic instability and prevents DNA damage-induced apoptosis. The pro-apoptotic translocation of Bax to mitochondria and the deamidation of Bcl_{XL} are inhibited, thus favouring survival of the CD45$^{-/-}$/Lck Y505F thymocytes. However, it is not yet known how hyperactive Lck Y505F mediates its effects on Bax/Bcl_{XL} signalling.

PTP-ε, another RPTP, also dephosphorylates SFKs, including Src, Yes and Fyn. It is not expressed in osteoblasts, but osteoclasts express a cytoplasmically localized splice isoform, cytPTP-ε. A. Elson (Rehovot, Israel) generated PTP-ε knockout mice and found that females younger than three months, but not male or older female mice, show an increase in trabecular bone volume because of reduced osteoclast function. Src$^{-/-}$ mice also show increased bone mass because of dysfunctional osteoclasts. However, although Src is activated by PTP-ε in some cell types, Src activity seems not to be affected in PTP-ε$^{-/-}$ osteoclasts. Elson also observed that the PTP-ε$^{-/-}$ osteoclasts have disorganized podosomes, which are structures that adhere osteoclasts to bone. Therefore, PTP-ε-mediated dephosphorylation is important for the correct positioning of podosomes and could account for the PTP-ε$^{-/-}$ phenotype observed. The gender dependence is ill-understood, but seems not to be directly hormonally regulated because the defects persist in isolated PTP-ε$^{-/-}$ osteoclasts.

RPTP-β/ζ and RPTP-γ are homologous RPTPs that are highly expressed in the nervous system in glial cells and in neurons, respectively. S. Harroch (Paris, France) reported that RPTP-β/ζ knockouts appear normal. However, in an experimental mouse model for autoimmune encephalitis (EAE), the RPTP-β/ζ$^{-/-}$ mice have a defect in myelin compaction and an increase in apoptosis of oligodendrocytes. Furthermore, RPTP-β/ζ is upregulated in remyelinating oligodendrocytes in human multiple sclerosis lesions, indicating that RPTP-β/ζ has an important role in oligodendrocyte survival.

Lafora disease is a fatal disorder that is characterized by progressive neurological deterioration, myoclonus and epilepsy. Patients accumulate intracellular Lafora bodies, which are polyglucosan inclusion bodies that consist of sparsely branched glycogen. S. Rodriguez de Cordoba (Madrid, Spain) found that in 65–70% of Lafora patients, *EPM2A* was mutated. This gene encodes laforin, a DSP that has a glycogen-binding site in its N terminus. All but one (G240S) of the point mutations that were

identified in patients abolished DSP activity and glycogen binding. Laforin binds to PPP1R5, a PP1-binding protein that contains binding sites for glycogen and glycogen synthase (GS). Interestingly, all the mutant laforin proteins from patients, including the mutant G240S that retains DSP activity and glycogen binding, bind poorly to PPP1R5, suggesting that the lack of interaction between this phosphatase and PPP1R5 has an important role in Lafora disease.

Reminiscent of the Lafora bodies are the β-amyloid plaques and neurofibrillary tangles (NFTs) composed of Aβ-42 and Tau protein that are observed in patients suffering from Alzheimer's disease (AD). Transgenic mice that express mutant Tau P301L (a common mutation associated with the development of AD) accumulate hyperphosphorylated Tau proteins in axonal structures as well as in the somata (J. Götz, Zurich, Switzerland). The injection of β-amyloid Aβ-42 fibrils into the brains of these transgenic mice leads to a five-fold increase in the number of NFTs. PP2A dephosphorylates Tau *in vitro* and *in vivo*, and interestingly, PP2A is downregulated in AD brains. The expression of a dominant-negative PP2A C-α L199P induces the specific hyperphosphorylation of Tau and the accumulation of Tau aggregates, indicating that PP2A has an important role in the development of AD.

Phosphatases in metabolism. Glycogen-metabolizing enzymes are tightly regulated by phosphorylation/dephosphorylation. P. Roach (Indianapolis, IN, USA) investigated which genes affect glycogen metabolism in *Saccharomyces cerevisiae*. Of the ~4,600 deletion mutants he tested (representing 80% of all genes), 242 of the mutants had high levels of glycogen, whereas in 324, this level was low. Among those with low glycogen were two that were mutated in the two regulatory targeting subunits *gac1* and *glc8* of the PP1-like Glc. These genes are known to be involved in glycogen metabolism and so validate the screen. Gac1 directs Glc7 to GS and is the yeast homologue of the regulatory PP1 subunit R_{GL}/G_M. Glc8 is related to the PP1 inhibitor, I-2. *glc7* itself was not identified in the screen because it is essential for yeast. Most other genes that were identified in the screen are linked to respiration or vacuolar transport.

In striated muscle, R_{GL}/G_M targets PP1 to glycogen. The *PPP1R3A* gene that encodes R_{GL}/G_M has been disrupted by homologous recombination by two independent groups. A. de Paoli-Roach (Indianapolis, IN, USA) found no significant difference in glucose tolerance, body weight, insulin responsiveness and abdominal fat levels of the R_{GL}/G_M knockout mice up to more than one year of age compared with wild-type mice. By contrast, P.T.W. Cohen (Dundee, UK) found decreased insulin-stimulated GS activity ratio in the absence or presence of glucose-6-phosphate in skeletal muscle, increased weight gain after three months, accumulation of abdominal fat, and development of glucose intolerance and insulin resistance at 11–12 months of age. The difference in phenotypes of the two R_{GL}/G_M knockout mice is not understood. The knockout strategy of the two groups is similar and no R_{GL}/G_M protein is detected in the homozygous mice. Genetic background might have a role, although normal insulin responsiveness in A. de Paoli-Roach's mice was observed in three independent knockout lines and in three different genetic backgrounds. De Paoli-Roach also reported that most of the muscle-GS knockout mice generated by P. Roach die of cardiac defects and an inability to take their first breath at birth. However, one in ten of the $GS^{-/-}$ mice survives. Their body weight is reduced, they have no GS or glycogen in their muscle but still their glucose tolerance is enhanced. Muscle glycogen accounts for only 0.3–0.7% of the daily caloric intake of mice and clearly, mice can survive with reduced GS activity ($R_{GL}/G_M^{-/-}$), or with no GS at all. Therefore, impaired glycogen accumulation in skeletal muscle does not seem to result in insulin resistance or obesity.

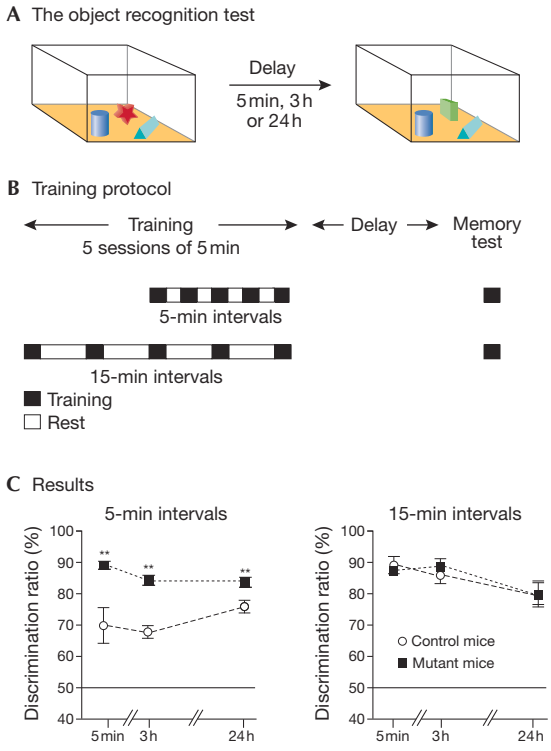

Fig. 4 | PP1 is a constraint on learning. (**A**) The object recognition test. Mice are repeatedly exposed to a set of objects (training); after a delay, one of the objects is exchanged for a foreign object and the mice are exposed again (test). The discrimination ratio is the time spent at the new object divided by the total time and is a direct measure for learning. (**B**) Training protocol. (**C**) I-1-expressing (mutant) mice learn significantly better with the 5-min interval than control mice (left panel). No difference was observed with the 15-min training interval (right panel) (courtesy of I. Mansuy, Zurich, Switzerland).

Phosphatases, behaviour and neuronal signalling. Phosphorylation has an important role in learning and memory. I. Mansuy (Zurich, Switzerland) developed an elegant system that allows doxycycline (dox)-inducible protein expression in the mouse brain. Importantly, gene expression is reversible by the removal of dox, so the experiment can be controlled. Transgenic mice that express the PP1-specific inhibitor, I-1, learn significantly better than control mice in a learning regime with 5-min inter-trial intervals, whereas there is no difference in learning with 15-min intervals (Fig. 4). Interestingly, endogenous PP1 activity in wild-type mice is decreased on the 15-min interval training regime. In I-1-expressing mice, the phosphorylation and activity of the

transcription factor cAMP-response-element binding protein (CREB) is enhanced in both the 5- and the 15-min interval regime, whereas in control mice only the 15-min intervals lead to CREB activation. PP1 inhibition also improves spatial learning, in that the I-1-expressing mice perform significantly better in the Morris water maze task, which correlates with increased Ca^{2+}/calmodulin-dependent kinase II and glutamate receptor phosphorylation. These results indicate that PP1 is a molecular constraint on learning and memory.

Not only Ser/Thr phosphorylation, but also Tyr phosphorylation might have a role in behaviour. W. Hendriks (Nijmegen, the Netherlands) generated mice in which the leukocyte antigen-related phosphatase (LAR) PTP domains are deleted (LAR-ΔP mice). Half of the homozygous LAR-ΔP embryos die around embroyonic day 16. The surviving mice show a reduced number and size of basal forebrain cholinergic neurons and reduced innervation of the supragranular and molecular layer. Learning and relearning is not affected in LAR-ΔP mice, but they are hyperactive in exploratory behaviour and nocturnal activity tests. How the deletion of LAR induces hyperactivity remains to be determined.

PPs are also important for neuronal development. In neurons, PP1 is highly concentrated in dendritic spines. S. Shenolikar (Durham, NC, USA) identified the evolutionarily conserved family of neurabins (Nrbs) as PP1-binding regulators in the spine. The Nrbs bind to F-actin through an N-terminal domain, which indicates that they have a role in actin cytoskeletal dynamics. NrbI induces filopodia formation in a PP1-dependent manner, and PP1 may dephosphorylate NrbI or other proteins in the NrbI–PP1 complex. The expression of Nrb1 in cultured rat hippocampal neurons induces spine formation and Shenolikar fused NrbI deletion mutants to green fluorescent protein to analyse the roles that the different regions of the protein have in this process. He found that the N-terminal region, NrbI (1–286) is required for initial morphological changes, NrbI (1–671) is important for spine development and full-length NrbI (1–1095) has a role in spine maturation.

Many PPs, particularly CN, are involved in neuronal signalling. A. Nairn (New Haven, CT, USA) found that PKA-mediated phosphorylation and activation of the PP1 inhibitor, DARPP32, is required for dopamine signalling-induced phosphorylation of the amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (AMPA-R). By contrast, AMPA and *N*-methyl-D-aspartate (NMDA) catalyse the dephosphorylation of the AMPA-R through the direct Ca^{2+}-dependent activation of CN, a process that does not involve DARPP32. In other studies, glutamate was found to induce a rapid, but transient, increase in the activation and nuclear translocation of MAPK, concomitant with Ca^{2+}-dependent CN-mediated dephosphorylation and activation of the striatal-enriched PTP, STEP, which limits the duration of MAPK activation and signalling. This dephosphorylation pathway, involving PP-mediated activation of a downstream PP by dephosphorylation, provides evidence for the existence of PP cascades.

Perspectives

PPs are tightly and dynamically regulated. Future work will reveal whether stimulus-induced inactivation of PPs, including oxidation-, phosphorylation- and inhibitor-induced inactivation, is actually required for signalling through phosphorylation. In addition, PP cascades that lead to amplification of the dephosphorylating potential or to a shift in substrate selectivity are likely to be found. Moreover, signalling complexes might be identified that contain a PP, a substrate and a PK, to facilitate very rapid changes in signalling. PP knockout mice and transgenic mice expressing PPs or their inhibitors have already provided starting points for the development of drugs to combat diseases, and ongoing analyses will provide more insight into the role of PPs in development and physiology. Finally, behaviour studies in transgenic animals, combined with the analysis of neuronal signalling will continue to provide valuable insights into the molecular basis of behaviour and learning.

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