Protein phosphatases, from molecules to networks

Anne-Claude Gingras

The third EMBO-sponsored 'Europhosphatases' meeting brought together 180 participants with a wide range of backgrounds and research interests to discuss the current status of research on phosphatases. It became clear at this meeting that the field is very active, and just as diverse as its members. This report highlights some of the transformative research presented at the meeting.

Phosphatase diversity

Introductory lectures from David Brautigan (U. Virginia, USA) and Nick Tonks (CSHL, USA) gave an historical insight into phosphatases. While the approximately 500 known protein kinases have a common ancestor and mechanism of catalysis, phosphatases are characterized by divergent evolution. The roughly 150 mammalian phosphatases can be broadly separated into gene families, including the cysteine-dependent protein tyrosine phosphatases (PTPs), the magnesium-dependent serine/threonine phosphatases (PPMs, also known as PP2C), the Ser/Thr-specific phosphoprotein phosphatases (PPPs), and the aspartate-based family. Subfamilies within these groups, and in particular for the PTPs, further add to the complexity.

While eukaryotic phosphatases are best characterized for their roles in dephosphorylating serine, threonine and tyrosine, other molecules can also serve as substrates. Jack Dixon (UC San Diego, USA) described PTPMT1, a dual specificity mitochondrial phosphatase essential for mitochondrial function and mouse development (Zhang *et al.*, 2011). Rather than dephosphorylating proteins, PTPMT1 acts as a lipid phosphatase, similarly to PTEN or myotubularin, and is responsible for the dephosphorylation of phosphatidylglycerol phosphate in the cardiolipin biosynthesis pathway. Other examples of phosphatases targeting non-protein substrates include the mRNA capping enzyme and laforin (a protein mutated in Lafora disease), which dephosphorylates glycogen. Dylan Silver from the Moorhead lab (U. Calgary, Canada) explained that

phosphatases analogous to laforin also exist in plants, where they are involved in starch degradation. Tim Clausen (IMP, Austria) described the discovery and characterization of the first arginine phosphatase, YwIE, in bacteria. Similarly to other PTP family members with altered substrate specificity, the preference of YwIE for a non-tyrosine substrate is conferred by amino acid substitutions in the catalytic pocket.

The diversity in phosphatase structures is paralleled by the variety of phosphate substrate recognition and regulatory mechanisms, which are relevant when considering therapeutic strategies. In-depth characterization by several participants of the functional roles of phosphatases in model organisms

revealed new roles for phosphatases in metabolism, neurological functions, immunity and cancer. A main theme of this meeting, however, was that a complete characterization of phosphatases as a family will require unbiased and systems-wide approaches: this is especially critical for poorly characterized phosphatases, many of which are associated with cancer in genomics studies (reviewed in Julien *et al.*, 2011). Due to space limitations, I focus here on global studies and provide a few specific examples. I apologize to all those whose work could not be included.

Phosphatase substrates and pathways

It has been difficult to systematically identify phosphatase substrates (although trapping mutants are successfully used for PTPs), but participants of this meeting highlighted a range of potentially useful new strategies. Anthony Bishop (Amherst College, USA) presented a chemical genetic approach using engineered PTPs that can be selectively inhibited by a cell permeable small molecule ligand (FlAsH). Thomas Kupka from the Ogris lab (MFPL, Austria) introduced M-TRACK, a modified yeast two-hybrid system designed for the identification of short-lived interactions, such as those between a phosphatase and its substrate. The system is based on the methylation of the histone H3 amino-terminus (fused to a 'prey') by a histone lysine methyltransferase (fused to a 'bait'): the specific accumulation of methylated H3-bait on interaction of a bait-prey pair is detected by methylation-specific antibodies.

The most frequently discussed screening approach was the use of RNA interference

(RNAi) screens. Jürgen Knoblich (IMBA, Austria) used a transgenic *Drosophila* RNAi collection to identify 620 proteins implicated in the control of asymmetrical division and stem cell self-renewal in neuroblasts, leading to the identification of the serine/threonine phosphatase PP4 (Neumüller *et al.*, 2011). Knoblich further demonstrated that PP4 is expressed in the ventricular zone of the developing mouse cortex, and that its downregulation results in misorientation of the mitotic spindle in neurons and premature neuronal differentiation at the expense of the neural progenitor pool. With other groups systematically analysing the effect of depletion of phosphatases on diverse readouts, including on growth pathways (Francesca Sacco, Cesareni lab, U. Rome, Italy), checkpoint recovery after DNA damage (Indra Shaltiel, Medema lab, UMC Utrecht, The Netherlands) and neurite outgrowth (Jan Sap, U. Paris Diderot, France), a more complete view of signalling pathways affected by phosphatases is emerging. When they are put in the context of parallel efforts in interaction proteomics, quantitative phosphoproteomics—as discussed by keynote speaker Matthias Mann (MPI of Biochemistry, Germany) and Anne-Claude Gingras (SLRI, Canada)—and *in vitro* phosphorylation analysis, these studies will undoubtedly shed light on the targets for several phosphatases.

How to regulate a phosphatase?

Far from being static and constitutively active enzymes, phosphatases are subject to tight regulation in a cellular context. Several mechanisms have evolved for the regulation of phosphatases, which differ for the various enzyme classes.

For PTPs, redox regulation was a significant component of this meeting: the catalytic cysteine of PTPs is particularly sensitive to oxidation, which serves as a reversible inhibitory switch. Soluble reactive oxygen species, including H_2O_2 , have previously been implicated as regulators of PTP oxidation. In this context, Åsa Sandin from the Östman lab (Karolinska Institutet, Sweden) showed that cellular lipid peroxides are regulators of PTPs. Tzu-Ching Meng (Academia Sinica, Taiwan) demonstrated that upon ischaemia—and in a model for myocardial infarction—PTPs are activated, which induces a global decrease in phosphorylated tyrosine accompanied by cytoskeletal changes. Treatment with nitric acid carrier S-nitroglutathione (GSNO) inactivates the PTPs and reverts the

cytoskeletal changes associated with ischaemia, suggesting potential therapeutic avenues. Ben Neel (Ontario Cancer Institute, Canada) presented a global quantitative proteomics approach to study reversible PTP oxidation that relies on an antibody specifically capturing the oxidized form of classical PTPs. One variation of the method allows the identification and quantification of all catalytically active PTPs from cells, whereas another modification allows the global assessment of different PTP oxidation states. Neel also showed the quantification of PTP oxidation levels across several cancer lines and demonstrated that the approach can be combined with tyrosine phosphorylation profiles to allow a systems-wide view of PTP signalling (Karisch *et al.*, 2011).

Several of the PPP family serine/threonine phosphatases associate with regulatory subunits, often in a combinatorial manner. This yields a large number of non-redundant holoenzymes, possibly to provide localization, substrate targeting or activation cues. Assembly of PP2A into holoenzymes also follows an ordered path, and several molecules have been implicated in this process. Claudia Stanzel from the Ogris lab showed that the yeast methylesterase Pme1 is implicated in a surveillance pathway for PP2A assembly, to prevent the release of prematurely carboxymethylated but inactive holoenzymes.

Protein inhibitors of PP1 and PP2A have long been known to be important for regulation of these enzymes. This year, a new family of PP2A inhibitors was reported to exquisitely control the PP2A-B55δ (*PPP2R2A*) holoenzyme to promote entry into mitosis. Mitotic entry requires precise regulation of the kinase-phosphatase balance: at the centre is cyclin-dependent kinase CDK1 activity, which is itself positively regulated by the Cdc25 phosphatase. PP2A antagonizes mitotic entry by dephosphorylating cyclin B-Cdk1 substrates. Anna Castro (CNRS, France) presented a feed-forward loop in *Xenopus* oocytes by which Cdk1 is required for activation of the Greatwall kinase (Gwl), which itself phosphorylates Arpp19 and Ensa. The phosphorylated forms of these molecules, but not their unphosphorylated species, serve as potent inhibitors of PP2A (Gharbi-Ayachi *et al.*, 2010). Regulation of entry into mitosis by several feedback loops enables bistability; the discovery of the Arpp19 loop enables systems modelling, as discussed by Bela Novak (U. Oxford, UK). An analogous system in mammals was presented by Angus Nairn (Yale U., USA), who discussed

the role of Arpp19 and the related Arpp16 and phosphorylation by the Gwl orthologue Mast3, but also by protein kinase A, in the mammalian brain.

No inhibitor of PP2Cs is known in the animal field, but a family of PP2C inhibitors has been discovered in plants. Erwin Grill (Technische U., Munich, Germany) and Pedro Rodriguez (CSIC, Spain) presented a family of receptors—the RCAR/PYR/PYL abscisic acid receptors—that interact with abscisic acid in response to stress (for example, drought) to inhibit the PP2C phosphatases. The structures of ternary complexes formed by receptor-abscisic acid-phosphatase uncovered the mechanism of action of the only known PP2C inhibitors (reviewed by Melcher *et al.*, 2010). PP2C inhibition induces activation of SNF1-related kinases, leading to modulation of the activity of their substrates—such as transcription factors, ion channels and the calcium-dependent kinase GCA2—which ultimately alters transcription and membrane permeability.

Kinase and phosphatase interplay

Kinases and phosphatases often play antagonizing roles in regulating a common substrate, but can also modulate each other. For example, David Brautigan showed that PP6 and casein kinase 1 oppose each other on the regulation of E-cadherin at cell-cell junctions, which adds to the number of functions attributed to this phosphatase in regulating protein kinases such as TAK1, DNA-PK and AurA. Rafael Pulido (Prince Felipe Research Centre, Spain) reported on the regulation of MKP3, which is phosphorylated by mitogen-activated protein kinases and casein kinase 2, resulting in its destabilization. Antje Gohla (U. Würzburg, Germany) reported interplay between the phosphatase chronophin and LIMK signalling in glioma cells.

Several studies also highlighted a molecular level understanding of cell cycle regulation or transcriptional activation through the concerted actions of kinases and phosphatases. Mathieu Bollen (U. Leuven, Belgium) discussed the human kinase haspin and a PP1 holoenzyme, both of which demonstrate exquisite specificity in targeting Thr3 of histone H3, but not all phosphorylation sites on this molecule. Patrick Hogan (La Jolla Institute for Allergy & Immunology, USA) showed that a cytoplasmic RNA-protein scaffold complex binds to the transcription factor nuclear factor of activated T cells (NFAT) and the kinases that keep it

inactive. Dephosphorylation of NFAT by calcineurin triggers dissociation of the complex and NFAT-dependent transcription. Frank Uhlmann (Cancer Research UK) presented a beautiful study monitoring the timing of the dephosphorylation events for many of the substrates of the Cdk1 kinase and Cdc14 phosphatase during exit from mitosis in yeast. Different substrates are dephosphorylated with different kinetics, a phenomenon that is related to the ratio of the Cdk1/Cdc14 activities. As quantitative proteomics and phosphoproteomics approaches become more widespread, it is expected that a systematic view of the kinase–phosphatase substrates relationships in space and time will emerge, enabling incorporation of dephosphorylation events into signalling models.

Phosphatases as therapeutic targets

Several participants, including keynote speaker Joseph Schlessinger (Yale School of Medicine, USA) and Stefan Knapp (Oxford U., UK), discussed in their talks or during question periods how kinases now constitute a favourite target for the pharmaceutical industry. By contrast, phosphatases are not generally considered ‘druggable’ by pharma. This is owing to several factors, including the similarity among catalytic sites, which renders specificity an issue, and the requirement for charged phosphate analogues to pass through the hydrophobic cell membrane to reach the targeted phosphatase. Meeting participants proposed several alternative strategies for generating better phosphatase inhibitors.

Zhong-Yin Zhang (Indiana U. School of Medicine, USA) discussed strategies to address the issues of specificity and bioavailability facing drug developers. Given the highly conserved nature of the PTP active site, Zhang is developing potent and selective inhibitors for individual members of the PTP family by tethering together small ligands that can simultaneously occupy both the active site and unique nearby peripheral binding sites for added specificity. These approaches, combined with others are allowing him to produce the first PTP inhibitors with both high affinity and selectivity, and excellent cellular efficacy.

Phosphatases such as PTP1B are regulated by reversible oxidation *in vivo*, and this modification induces profound conformational changes in the proteins. Nick Tonks raised single chain variable fragments (scFvs) to a conformational mimetic of the oxidized enzyme. Used intracellularly, these

oxidation-specific scFvs sequester oxidized PTP1B and inhibit the reduction and reactivation of the oxidized enzyme, thereby functioning as inhibitors of the phosphatase. Consistent with the crucial role of PTP1B in insulin signalling, expression of the scFvs led to enhanced and sustained insulin signalling, suggesting that small molecules mimicking the effects of these scFvs would represent a new strategy for therapeutic intervention in diabetes and obesity (Haque *et al*, 2011).

In the case of the members of the PPP family that form potentially hundreds of different holoenzymes (these include PP1 and PP2A), it would be important to target a specific holoenzyme rather than the entire phosphatase pool. Clearly, the increased knowledge of the structural organization of PP1 and PP2A in complex with regulatory proteins and substrates could help achieve this goal. Wolfgang Peti (Brown U., USA) presented several structures of the PP1 catalytic subunit in association with different binding partners. Interestingly, his studies have revealed that although many of the interaction partners establish interactions with PP1 through shared surfaces, each interactor also establishes specific extended contacts with other areas on the catalytic subunit. This is exciting, as it provides a target for prevention of specific complex assembly.

An alternative approach to inactivate a specific holoenzyme would be to directly target the regulatory subunit. This might prevent association with the catalytic subunit, preclude association with a substrate, or target the regulatory subunit for degradation. However, until recently, there was no example of a specific ‘druggable’ catalytic–regulatory complex. This year, Anne Bertolotti’s group (MRC LMB, UK) reported that the small molecule guanabenz targets the GADD34 subunit of PP1 (Tsaytler *et al*, 2011). As discussed by Bertolotti and Shirish Shenolikar (Duke–NUS, Singapore), GADD34 associates with PP1 to specifically dephosphorylate the translation initiation factor eIF2 α . Under endoplasmic reticulum stress, phosphorylation of eIF2 α at a single site results in translational inhibition: this inhibition is important to allow sufficient time for effective protein folding by available chaperones, thereby maintaining proteostasis. Importantly, some mRNAs escape this inhibition, including those encoding the transcription factor CHOP and its downstream target GADD34. Therefore, endoplasmic reticulum stress leads to an increase in the PP1•GADD34 phosphatase, creating

a feedback loop to restore translation. Bertolotti’s findings demonstrate that only the stress-induced GADD34 protein, but not its closest relative—a constitutively expressed PP1 regulatory subunit encoded by the gene *PPP1R15B*—can be targeted by guanabenz. This is in fact the second such example of a small molecule inhibitor of an eIF2 α phosphatase, but the first to demonstrate such specificity.

Conclusions

Inhibition of PP1 holoenzymes demonstrates that regulatory subunits can be inhibited and constitute potentially important drug targets. However, it is also clear that a much better characterization of the mechanisms for regulatory subunit assembly and the functional role of each holoenzyme will be necessary for rational drug design. Inhibition is also not always the desired outcome: Dale Christensen (Duke U., USA) reported that using cell-penetrating peptides that antagonize the PP2A inhibitor SET—which is upregulated in several cancers—leads to sustained PP2A activation and decreased cancer progression.

Advances presented by the meeting participants that combine genetic or substrate screening, in-depth functional analysis and structural determination are beginning to shed light on the multiple roles of phosphatases and lay the foundation for better therapeutics.

ACKNOWLEDGEMENTS

I am grateful to the meeting’s organizer, Egon Ogris (MFPL, Austria), and co-organizers Irute Meskiene (MFPL, Austria), Jeroen den Hertog (Hubrecht Institute, The Netherlands) and Ari Elson (Weizmann Institute of Science, Israel), as well as to colleagues who gave permission to share their work and who read the manuscript.

REFERENCES

- Gharbi-Ayachi A *et al* (2011) *Science* **330**: 1673–1677
- Haque A *et al* (2011) *Cell* **147**: 185–198
- Julien SG *et al* (2011) *Nat Rev Cancer* **11**: 35–49
- Karisch R *et al* (2011) *Cell* **146**: 826–840
- Melcher K *et al* (2010) *Curr Opin Struct Biol* **20**: 722–729
- Neumüller RA *et al* (2011) *Cell Stem Cell* **8**: 580–593
- Tsaytler P *et al* (2011) *Science* **332**: 91–94
- Zhang J *et al* (2011) *Cell Metab* **13**: 690–700

**Anne-Claude Gingras is at the Samuel Lunenfeld Research Institute and the University of Toronto, Canada.
E-mail: gingras@lunenfeld.ca**

Published online 18 November 2011

EMBO reports (2011) **12**, 1211–1213.
doi:10.1038/embo.2011.225