REVIEWS

Emerging roles of nuclear protein phosphatases

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Abstract | The phosphorylation state of any protein represents a balance of the actions of specific protein kinases and protein phosphatases. Many protein phosphatases are highly enriched in, or exclusive to, the nuclear compartment, where they dephosphorylate key substrates to regulate various nuclear processes. In this review we will discuss recent findings that define the role of nuclear protein phosphatases in controlling transforming growth factor-β (TGFβ) and bone-morphogenetic protein (BMP) signalling, the DNA-damage response, RNA processing, cell-cycle progression and gene transcription.

Protein phosphorylation is recognized as a widespread and highly versatile regulatory mechanism that controls many cellular processes, and recent quantitative phosphoproteomics have indicated that phosphorylation preferentially targets nuclear proteins[1]. Aberrant protein phosphorylation has been linked to many human diseases, and, as a consequence, the well studied protein kinases are currently the pharmaceutical industry's second largest drug target[2]. By contrast, protein phosphatases are just beginning to be recognized as future therapeutic targets[3–5].

The protein phosphatases that regulate nuclear events are often enriched in, and sometimes exclusively localized to, the nucleus. Yet until recently, few were well characterized. This is partly due to the limitations of the available reagents, such as the broad specificity of many protein phosphatase inhibitors. For example, the functions of certain phosphatase families are defined by additional regulatory or interacting proteins that bind the catalytic subunits, target them to specific locations and/or substrates and control their activity. Inhibitors that target the catalytic subunit therefore inhibit a wide range of holoenzyme phosphatase complexes that are involved in a diversity of signalling pathways. The identification and characterization of phosphatase regulatory proteins by various methods is helping us to define the holoenzyme complexes and to develop techniques to disrupt specific phosphatase–substrate interactions.

The importance of protein phosphatases was demonstrated many years ago when it was shown that they are the target of many naturally occurring toxins, they function as tumour suppressors and they are frequently the target of viral proteins, including the viral oncoprotein E4orf4 and the middle-T and small-T antigens[6,7]. More recently, large-scale loss-of-function RNA interference (RNAi) screens have revealed that a surprising number of phosphatases, many of which function in the cell nucleus, are previously unrecognized promoters of both cell survival and cell death[8], and that many have pivotal roles in the regulation of cell-cycle progression[9].

Genomics studies have produced a catalogue of protein kinases and phosphatases for several eukaryotic organisms, including humans (BOX 1), and have therefore provided the means to develop a complete inventory of phosphatase tools for large screens. This type of approach has resulted in a flurry of new insights on nuclear protein phosphatase function in a diversity of nuclear processes, some of which we will discuss here. Before highlighting these new studies, we will provide a framework to understand the large (and growing) human protein phosphatase repertoire as a whole, detailing protein phosphatases that function in the nucleus.

Classification of protein phosphatases
The protein phosphatases were initially divided into families primarily on the basis of whether they could dephosphorylate Ser or Thr (Ser/Thr) residues alone, Tyr residues alone, or whether they had dual specificity. Initially, gene sequencing and genomic analysis maintained these borders of separation, but we now know that certain Ser/Thr-specific enzymes can dephosphorylate Tyr, and that many of the enzymes that fall into the dual-specificity group, on the basis of sequence, can selectively function on Tyr, Ser, Thr, RNA or phosphoinositides[10–12]. More recently, several members of the haloacid dehalogenase (HAD) superfamily were shown to have protein phosphatase activity and to dephosphorylate Ser or Tyr[13,14]. These enzymes use an Asp nucleophile for catalysis and carry the active-site signature DXDXT/V, just like the previously characterized TFIIF-associating C-terminal domain (CTD)

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SILAC
A mass-spectrometry-based quantitative proteomics method that uses stable-isotope labelling with amino acids in cell culture.

phosphatase-1 (FCP1) and small CTD phosphatase (SCP) enzymes, which dephosphorylate the CTD of the largest subunit of RNA polymerase II (Pol II)[5,15–17].

Protein phosphatases can be classified into three groups on the basis of sequence, structure and catalytic mechanism. The first group comprises the classic Ser/Thr phosphatases: the large phosphoprotein phosphatase (PPP) family (PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7) and the protein phosphatase, Mg^{2+} or Mn^{2+} dependent (PPM) family (PP2C). The protein Tyr phosphatase (PTP) superfamily forms the second group, and the third group consists of the Asp-based protein phosphatases with a DXDXT/V catalytic signature.

Protein Ser/Thr phosphatases

Protein Ser/Thr phosphatase activity in mammalian extracts was initially defined using biochemical assays and was classified as either type 1 (PP1) or type 2 (PP2), and was further subdivided into categories on the basis of metal-ion requirements (PP2A, no metals; PP2B, Ca^{2+} stimulated; PP2C, Mg^{2+} dependent) and substrate specificity. PCR-based cloning and genomics uncovered the complement of related phosphatases (PP4–7) and the likely evolutionary history of these enzymes. Enzymes that display protein Ser/Thr phosphatase activity, with a few exceptions from the PTP family and the Asp-based family as discussed below, belong to the PPP and PPM families (TABLE 1).

PP1. The type 1 protein phosphatases are encoded by three highly related genes in humans (*PP1α*, *PP1β/δ* and *PP1γ*), and alternative splicing generates the γ1 and γ2 isoforms. With the exception of *Saccharomyces cerevisiae*, which has 1 PP1 gene (*glc7*), all eukaryotes have multiple PP1 genes, with 8 in *Arabidopsis thaliana*, 4 in *Drosophila melanogaster* and a predicted 30 in *Caenorhabditis elegans*[18].

Although the cytosolic forms of mammalian PP1, such as those targeted to glycogen and myosin, have been studied in greatest detail, PP1 activity is enriched in the nucleus[19,20]. The significance of the different PP1 isoforms remains unclear; however, *in vivo* data show that they have distinct subcellular localization patterns[21,22]. All isoforms are found in the nucleus in interphase cells, with PP1γ and PP1β/δ showing additional accumulations in nucleoli[22,23]. PP1-isoform localization patterns are also dynamic and change both throughout the cell cycle and in response to various cellular perturbations (FIG. 1a). It is important to note that the global patterns of localization that are observed for each isoform represent the sum of many different PP1–targeting-subunit complexes (FIG. 1b), as shown by the immunostaining of several nuclear targeting subunits with overlapping localization patterns (FIG. 1C). The distinct localization patterns of the PP1 isoforms therefore imply differences in the specificity of interaction with particular targeting subunits, and so preferential incorporation into different signalling complexes.

With few exceptions so far, PP1 associates with its targeting subunits through the small motif RVXF. This is thought to be the primary PP1-docking site, with secondary sites contributing to the interaction to various extents[24–29]. There are now more than 90 documented PP1-targeting subunits with this signature and/or with demonstrated docking through the RVXF motif, and many more remain to be discovered. Examination of the motif in characterized PP1-interacting proteins, coupled with studies directed at refining this motif, have defined the motif as $R/K_{1-2}V/I[P]F/W$, for which any amino acid can occupy the site in brackets except Pro[30]. A recent in-depth study of the motif further refined this docking site, which was subsequently used as a bioinformatics tool to predict 84 new PP1-interacting proteins that are conserved in mammalian genomes[31]; 7 out of the 8 predicted PP1-interacting proteins that were tested were confirmed to bind PP1 *in vitro*. It should be noted that, even though this refined docking site reduces the number of false hits, it does not recognize several previously identified and characterized PP1 interactors, such as inhibitor-1 and myosin phosphatase-targeting subunit-1 (MYPT1).

Initial fractionation studies using anion-exchange chromatography revealed two major nuclear forms of mammalian PP1 — NIPP1 and p99 (also known as PNUTS)[32] — and subsequent studies have steadily added to this growing list of nuclear targeting subunits[32–37]. Proteomics approaches have also been useful for identifying new nuclear PP1-targeting subunits[33]. Recently, a powerful combination of time-lapse fluorescence microscopy and quantitative mass-spectrometry-based proteomics (the SILAC technique[38]) was used not only to identify known and new PP1-interacting proteins, but also to determine their differential binding to the α- and γ-isoforms of PP1 (REF. 34). Combining these identified isoform preferences with the observation that a pool of PP1γ specifically accumulates on chromatin during anaphase and remains associated with it throughout interphase led to the identification of Repo-Man, a new

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Box 1 | **Human protein kinases and phosphatases**

Proteins are phosphorylated predominantly on Ser, Thr and Tyr residues, with each accounting for approximately 86.4, 11.8 and 1.8%, respectively, of the human phosphoproteome[1]. Genomics studies have provided a catalogue of protein kinases and phosphatases for several eukaryotic organisms. Human DNA encodes 518 protein kinases, with 428 known or predicted to phosphorylate Ser and Thr residues and 90 belonging to the tyrosine kinase family[10,114]. By contrast, there are only ~147 human protein phosphatase catalytic subunits, with 107 belonging to the Tyr phosphatase family[10,115,116]. Of these 107 catalytic subunits, 81 are predicted to be active protein phosphatases[10,116]. Interestingly, although more than 98% of protein-phosphorylation events take place on Ser and Thr side chains, only ~40 of the 147 known protein phosphatases are specific for these amino acids. The large number of tyrosine phosphatases have evolved by adding discrete modular domains onto a core catalytic subunit, and it is these domains that define the functions of an enzyme[3,10]. Comparatively few Ser/Thr catalytic subunits exist, and this discrepancy can be explained by the unique manner in which they are regulated. Of these ~40 phosphatases, 18 are PP2C enzymes that do not have additional regulatory subunits. For the remaining Ser/Thr phosphatases, function is defined by additional regulatory or interacting proteins that bind the phosphatase catalytic subunits (and holoenzymes), target them to specific locations and/or substrates and control their activity. One of the major advances in protein phosphatase research over the past few years has been the ongoing identification and characterization of proteins that harness the enzymatic activity of the catalytic subunits in this way for many roles throughout the cell.

Table 1 | **Classification of human protein phosphatases**

Family	Class	Number of genes	Regulatory subunits	Example of function and/or (substrate)	References
Ser/ Thr phosphatases					
PPP family	PP1	3	>90 (e.g., Repo-Man)	Chromosome condensation	34, 39
	PP2A	2	A, B‡, etc.§	Chromatid cohesion	78
	PP4	1	R1, R2, R3α/β, etc.¶	DNA repair (γ-H2AX)	44, 94, 95
	PP5	1	None	Cellular stress	53
	PP6	1	SAP1–3, etc.¶	NFκB pathway	52
	PP2B	3	Regulatory B, CaM	Immune response (NFAT)	117
	PP7	2	Unknown		
PPM family	PP2C	18	None	TGFβ signalling (SMADs)	105, 111, 112
PTP superfamily (CX₅R)					
Class I PTPs (classic*)	Receptor PTP	21		Cell adhesion/cytoskeletal	3
	Non-receptor PTP	17		Insulin signalling (insulin receptor)	118
Class I PTPs (DSPs)	MAPKP	11		MAPK signalling (MAPK)	3, 10
	Slingshots	3		Actin dynamics (cofilin)	119
	PRLs	3		Unknown	3, 10
	Atypical DSP	19		Mostly unknown (mRNA)	65
	CDC14	4		Cytokinesis, mitotic exit	82
	PTEN	5		PIP₃ phosphatase	120
	Myotubularins	16		PtdIns3P, PtdIns(3,5)P₂ phosphatase	11, 120
Class II PTPs	CDC25s	3		Promotes mitosis (CDKs)	82
Class III PTPs	LMWPTP	1		Unknown	10
Asp-based catalysis (DXDXT/V)					
FCP/SCP family	FCP1	1	RAP74 of TFIIF	Transcription (Pol II)	15
	SCP	3		TGFβ signalling (SMADs)	108, 109
	FCP/SCP-like**	4		Unknown	5
HAD family		5‡‡		Actin dynamics (cofilin)	75

*Note that the division of receptor and non-transmembrane (non-receptor) is not absolute as the use of alternative splicing and promoters can generate both forms from one gene[3]. ‡Note that the B class of PP2A subunits includes B, B′, B″ and B‴ (see main text). §Additional PP2A C catalytic subunit-binding proteins are known, including α4 (also known as IGBP1; Tap42 in yeast) and Tip41 (see main text). ¶Additional PP4- and PP6-interacting proteins are known, including α4 and TIP41 (see main text). **Additional members with FCP1-like catalytic domains include Dullard, HSPC129, TIMM50 and MGC10067. ‡‡Even though this is a large superfamily of enzymes, so far only chronophin and Eya1–4 have been shown to display protein phosphatase activity. CaM, calmodulin; CDK, cyclin-dependent kinase; DSP, dual-specificity phosphatase; Eya, Eyes Absent; FCP, TFIIF-associating C-terminal domain phosphatase; H2AX, histone 2AX; HAD, haloacid dehalogenase; LMWPTP, low-molecular-weight PTP; MAPK, mitogen-activated protein kinase; MAPKP, MAPK phosphatase; NFκB, nuclear factor κB; NFAT, nuclear factor of activated T-cells; PIP₃, phosphatidylinositol-3,4,5-trisphosphate; Pol II, RNA polymerase II; PPM, protein phosphatase, Mg²⁺ or Mn²⁺ dependent; PPP, phosphoprotein phosphatase; PRL, phosphatase of regenerating liver; PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; PTEN, phosphatase and tensin homologue; PTP, protein Tyr phosphatase; SAP, Sit4-associated protein; SCP, small C-terminal domain phosphatase; Tap42, 2A phosphatase-associated protein of 42 kDa; Tip41, Tap42-interacting protein; TGFβ, transforming growth factor-β.

HEAT repeat
A tandemly repeated module, 37–47 amino acids in length, that is present in a number of proteins, including the four proteins that gave the module its name: huntingtin, elongation factor-3 (EF3), the A subunit of PP2A and target of rapamycin kinase-1 (TOR1).

TOR signalling
Signal-transduction events that are mediated through the target of rapamycin (TOR) protein complexes.

nuclear targeting subunit that mediates the regulated interaction of PP1 with chromatin throughout the cell cycle[34]. The Repo-Man–PP1 complex is essential for cell viability and has recently been shown to participate in the maintenance of chromosome architecture during segregation[39].

PP2A, PP4 and PP6. PP2A exists as either a heterodimer or heterotrimer in cells. The HEAT-repeat-containing A subunit forms a horseshoe-shaped scaffold to tether the catalytic C subunit (PP2Ac) by its unique C-terminal tail[40], which explains why the A subunit only binds PP2Ac and no other PPP enzymes. The structure of a trimeric PP2A complex contains, in addition to

Aα and Cα, the B56γ₁ subunit, which interacts with the C subunit near the active site, thereby defining substrate specificity[41,42]. The C subunit can also bind α4 (also known as IGBP1; the mammalian orthologue of yeast 2A phosphatase-associated protein of 42 kDa (Tap42)) and Tap42-interacting protein (Tip41), which controls TOR signalling[43,44]. Mammals have two PP2A catalytic subunit genes (*PPP2Cα* and *PPP2Cβ*) and two A subunit genes (*PR65α* and *PR65β*). There are four classes of B subunits with multiple genes in each group: the B family (also known as B55, PR55 or PPP2R2), the B′ family (also known as B56, PR61 or PPP2R5), the B″ family (also known as PR72 or PPP2R3) and the B‴ family (PR93/SG2NA and PR110/striatin).

a | Interphase | Mitosis

GFP–PP1α

GFP–PP1γ

b

— Unidentified nuclear body

— Cajal body

— Nucleolus

— Nuclear speckle

Nuclear lamina

PP1   AKAP149   p99/PNUTS   ?

NIPP1   ZAP   ?
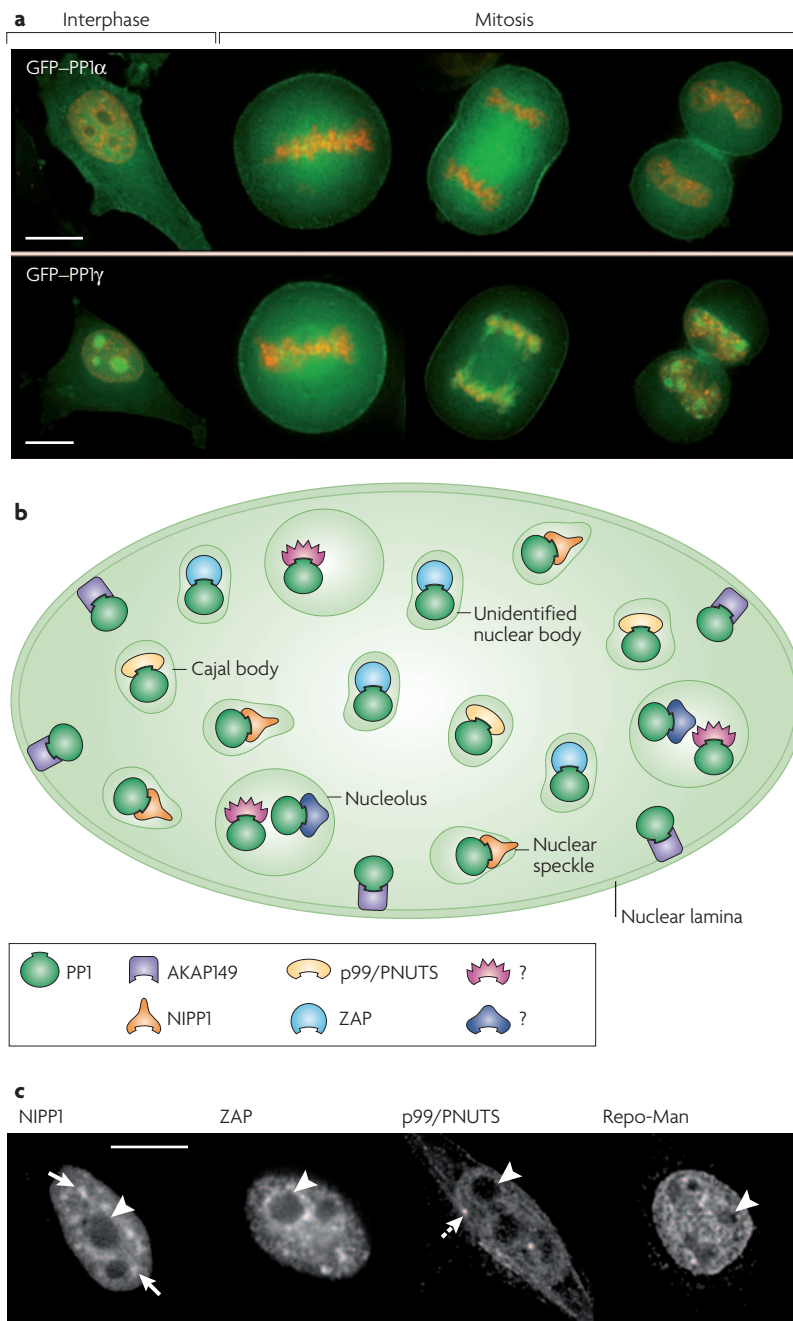
c

NIPP1   ZAP   p99/PNUTS   Repo-Man

Figure 1 | **The localization and activity of PP1 are regulated by a pool of targeting subunits. a** | Two different human isoforms of PP1 show distinct and dynamic subcellular localization patterns, both in interphase and during cell division. Green fluorescent protein (GFP)-tagged PP1α and PP1γ, stably expressed in HeLa cells, are shown in green, and DNA stained with Hoechst 33342 is shown in red. Images are single 0.5 μm sections taken through the centre of a live cell. Scale bars are 10 μm. **b** | The global subnuclear localization pattern of PP1 observed during interphase is the sum of the localization of various pools of PP1-targeting subunit complexes, including PP1–AKAP149 (nuclear lamina), PP1–NIPP1 (nuclear speckles), PP1–ZAP (unidentified nuclear bodies) and PP1–p99 (also known as PNUTS) (Cajal bodies). **c** | Many nuclear targeting subunits identified so far show similar subnuclear localization patterns, with a diffuse nucleoplasmic background and some differences in localized accumulations. Paraformaldehyde-fixed HeLa cells were stained with antibodies to NIPP1, ZAP, p99/PNUTS and Repo-Man. Nucleoli are indicated by arrowheads in each cell, accumulations of NIPP1 at nuclear speckles are indicated with arrows and an accumulation of p99/PNUTS at a Cajal body is indicated with a hashed arrow. Scale bar is 10 μm.

Several studies have addressed the cellular localization of PP2A. Early work by Turowski *et al.* revealed that the A and C subunits of PP2A were approximately twofold more concentrated in the nucleus than in the cytoplasm and did not reside in nucleoli[45]. Whereas B55δ, B56α, B56β and B56ε are cytosolic, other B55 subunits, B56δ and B56γ are highly enriched in the nucleus, and the CDC6 interactors PR48 and PR90 are exclusively nuclear[46–48]. The viral E4orf4 protein has been shown to target nuclear PP2A by binding the B55α and B56 subunits[49]. In *S. cerevisiae*, PP2A subunits display dynamic localization patterns, with both catalytic subunits (Pph21 and Pph22) and the A (Tpd3), B (Cdc55), B′ (Rts1) and putative B′ (Rts3) subunits showing at least partial nuclear localization[50].

PP4 and PP6 are most closely related to PP2Ac and also interact with α4 (also known as IGBP1; or Tap42 in yeast) and Tip41 (REFS 44,51). PP6 (or Sit4 in yeast) has several related binding partners, which are referred to as Sit4-associated proteins (SAPs), that are conserved in other eukaryotes[52]. The yeast PP6 protein is both nuclear and cytoplasmic.

The past year has resulted in an enormous advance in our understanding of the role of PP4 in the DNA-damage response (see below) and in identifying PP4-regulatory subunits and PP4-binding proteins. Gingras *et al.* stably expressed a tandem affinity-purification tagged (TAP-tag) PP4 catalytic subunit in mammalian cells to look for binding partners[44]. Purified components identified by liquid-chromatography–tandem-mass-spectrometry (LC–MS/MS) included the previously known R1 and R2 subunits, as well as α4, the eight-subunit ATP-dependent chaperonin complex TRiC (also known as CCT) and two related new proteins, which have since been designated R3α and R3β. By TAP-tagging each of these proteins, carrying out pull-downs and identifying the binding partners, it was established that PP4 resides in three complexes: with R1, with α4 and the TRiC/CCT proteins, and with R2 and R3, which might also contain Tip41. Orthologues of each of these proteins exist in the *S. cerevisiae* genome, and this work, along with complementary large-scale interaction data sets, confirms that they are in similar complexes to those in human cells. Yeast R2 (Psy4) and R3 (Psy2) proteins are exclusively nuclear, whereas PP4 (Pph3) is nuclear and cytoplasmic.

PP5. PP5 is unique in that it has a series of three N-terminal TPR domains that function to keep the enzyme inactive. Structural analysis of human PP5 showed that the mechanism of TPR inhibition of the phosphatase domain is similar to the mechanism of inhibition of PP2B by its C-terminal regulatory segment and to that of PP1 by toxins[53]. The enzyme is cytoplasmic and nuclear in both humans and *S. cerevisiae*, and the C terminus contains a conserved sequence that is required for nuclear localization[54,55].

PP2B and PP7. These enzymes are grouped together because they are stimulated by Ca^{2+}. PP2B, also known as PP3 or calcineurin, is a dimer that consists of a catalytic A subunit and a separate B subunit that is related

to calmodulin. PP7 has EF-hand domains and is highly orthologous to the *D. melanogaster* protein retinal degeneration C phosphatase[56]. Although a small population of PP2B resides in the nucleus, little is known about its role there. The *A. thaliana* PP7 is almost exclusively localized in the nucleus and is thought to regulate blue-light signalling[57].

The PPM family. Comparison of the crystal structure of the PPM-family protein PP2Cα with that of PPP-family members shows that the active sites of the two families are remarkably similar, although the families do not share any sequence homology[58]. There are at least 18 PP2C genes in humans and a predicted 7 in *S. cerevisiae*, but there are an astounding 76 PP2Cs in *A. thaliana*, and these encode various modular domains as N- and C-terminal extensions that function to localize the enzymes to specific locations and/or substrates[59]. A number of human PP2Cs have been localized almost exclusively to the nucleus (PP2Cα, PP2Cδ and PP2Cε), and PP2Cγ, PP2Cκ and PP2Cβ have significant nuclear localization[60–62]. PP2Cγ was previously found as a component of the spliceosome and was characterized as a pre-mRNA splicing factor[63,64].

Protein Tyr phosphatases
The PTP superfamily was defined on the basis of the catalytic CX_5R signature, which was derived from the first PTP purified; however, substrates now include phosphorylated Ser and Thr, mRNA and phosphoinositides[3,10,11,65]. The PTP superfamily is further divided into evolutionarily distinct families on the basis of gene sequence and structure (TABLE 1).

Of the classic non-receptor PTPs of class I, several have been characterized as being localized in the nucleus and include PTP36, PEP-PTP, SH-PTP1, PTP-S, PTP-BL, SHP-1, TCPTP (also known as TC45) and PTPε[66–73]. The *PTPε* gene generates four alternative transcripts, only one of which has an N-terminal extension that confers nuclear translocation[73]. TCPTP resides in the nucleus until it is shuttled to the cytoplasm in response to epidermal-growth-factor receptor (EGFR) activation, where it recognizes the EGFR and the adaptor protein p52shc as cellular substrates[70]. Metabolic and drug-induced activation of the AMP-activated protein kinase was the only stress found to cause the accumulation of TCPTP in the cytoplasm and therefore to regulate TCPTP function *in vivo*.

Asp-based protein phosphatases
Another group of protein Ser/Thr phosphatases has been identified, with the founding member, FCP1, initially purified as the nuclearly localized phosphatase that is responsible for the dephosphorylation of the CTD of the largest subunit of Pol II[74]. This class now includes the related nuclearly localized SCP enzymes, which lack the BRCT domain present in FCP1 (REFS 15,16), and four other enzymes (TABLE 1).

The second class of Asp-based protein phosphatases comprises the HAD superfamily. Recently, attention has been drawn to several HAD-superfamily members, such

as chronophin, which controls cofilin phosphorylation and therefore actin-cytoskeleton dynamics, and Eyes Absent (Eya), which functions as a transcription factor in the nucleus[13,14,75]. Like the FCP and SCP enzymes, HAD-superfamily members have a DXDXT/V catalytic signature and use a unique Asp-based catalytic mechanism[15]. So far, only a few HAD-superfamily members have been demonstrated to display Ser or Tyr phosphatase activity.

Phosphatase functions in the nucleus
The nucleus is a highly dynamic cellular compartment where protein phosphorylation has a dominant regulatory role[1]. The application of phosphatase inhibitors, genetics and, more recently, large-scale RNAi and overexpression screens has linked protein phosphatase function to a multitude of nuclear processes as diverse as DNA replication and repair, chromosome condensation, ribosome biogenesis, chromatin remodelling and various signal-transduction pathways[76,77]. Also, many intra- and extracellular signalling pathways, including those involved in programmed cell death and apoptosis, ultimately converge on the nucleus. Although we cannot discuss the role of protein phosphatases in all of these nuclear events, we will highlight several processes for which advances have recently been made.

For example, the complex process of cell division involves multiple independent regulatory steps, most of which are controlled by reversible protein phosphorylation. The importance of regulated protein phosphatase activity in the control of cell-cycle progression is at last being appreciated, largely owing to recent studies linking phosphatases to crucial regulatory events. The PTPs are the best characterized so far; CDC25 is known to be a key regulator of initiation of mitosis, whereas CDC14 is involved in later mitotic events. In addition, PP1 and PP2A have both been recently shown to have important roles in the regulation of chromosome segregation[39,78–81]. For a more comprehensive review of mitotic protein phosphatases, see REF. 82.

DNA-damage response
In response to damaged DNA, cells undergo a transient cell-cycle arrest to allow the DNA-repair machinery to fix the genomic DNA. This is a rapid, elaborate and synchronized event that involves many repair proteins and protein-phosphorylation events.

An integrated role for PP2C. The PP2C-family member PP2Cδ (also known as WIP1 or PPM1D) is unique in that its transcription is induced in response to DNA-damaging agents in a p53-dependent manner, and the enzyme resides almost exclusively in the nucleus[83]. The first identified target of PP2Cδ was the stress-response kinase p38 mitogen-activated protein kinase (MAPK), which is dephosphorylated by PP2Cδ on the Thr residue of the activation loop's TXY motif[84]. This effect was postulated to abrogate the ultraviolet (UV)-induced activation of p38, which normally activates p53 by phosphorylation, thereby forming a negative-feedback loop for p53. An additional study indicated that another nuclear PP2C, PP2Cα, negatively regulates the human
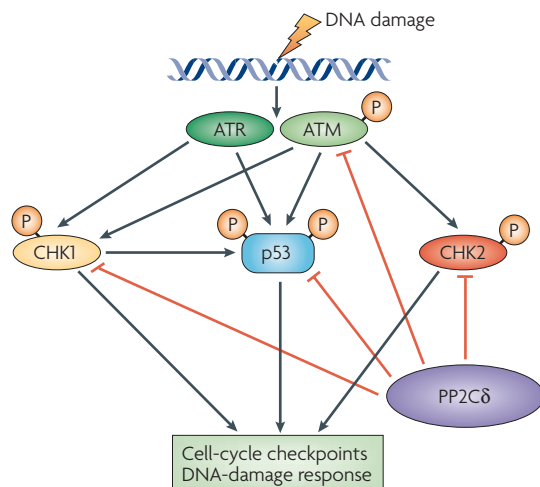

Figure 2 | PP2Cδ and the DNA-damage response.
In response to DNA damage, cells initiate a programme of cell-cycle checkpoints and DNA repair. DNA damage activates the PIKK-like protein kinases ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR), which, in turn, phosphorylate and activate many downstream targets, including p53, checkpoint kinase-1 (CHK1) and CHK2. A series of studies have now shown that the nuclearly localized phosphatase PP2Cδ (also known as WIP1) is a key integrator of the response, which attenuates signalling through the ATM and ATR pathways. It is thought that PP2Cδ interacts directly with ATM, p53, CHK1 and CHK2 and dephosphorylates these targets. P, phosphate.

stress-responsive p38 and JNK MAPK pathways by dephosphorylating both MAPK kinase (specifically, MKK6 and SEK1) and p38 MAPK[85].

A recent report showed that PP2Cδ also dephosphorylates the nuclear base-excision-repair protein uracil DNA glycosylase (UNG2) on the Thr residue of another TXY motif, thereby suppressing enzyme activity[86]. PP2Cδ prefers to dephosphorylate the Thr or Ser residues of dual-phosphorylated over mono-phosphorylated motifs, as shown by detailed kinetic studies[87].

Another key player in this regulatory pathway is the checkpoint kinase-1 (CHK1) protein kinase, which is activated by the DNA-damage-response protein kinases ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) (FIG. 2). CHK1 directly binds PP2Cδ and is dephosphorylated and inactivated by the PP2C enzyme[88]. It is interesting to note that a plant PP2C, known as abscisic acid insensitive-2 (ABI2), has been shown to interact with a group of plant protein kinases[89]. A 32-amino-acid fragment in these kinases was found to bind PP2C, and a very similar region was detected in the protein kinase CHK1. This segment of CHK1 is conserved across many organisms, including humans.

The discovery that a phospho-SQ motif in CHK1 is a substrate for PP2Cδ prompted a similar investigation of p53, which is itself an ATM/ATR substrate. Indeed, p53 was also shown to be a PP2Cδ target[88] (FIG. 2). Overexpression of PP2Cδ in irradiated or UV-treated cells abolishes the intra-S and G2–M cell-cycle checkpoints in response to DNA damage, whereas repression of PP2Cδ prolongs these checkpoints.

The integration of PP2Cδ into the DNA-damage response has been further strengthened by the observation that PP2Cδ also binds and inactivates the other human checkpoint kinase, CHK2, in the nucleus (FIG. 2)[90]. The direct binding of a PP2C to CHK2 is conserved in yeast; the S. *cerevisiae* homologue of CHK2 (Rad53) and PP2C (Ptc2 and Ptc3) had previously been shown to interact[91]. A further connection between PP2C and DNA damage was established when it was shown that PP2Cδ controls the phosphorylation of Ser1981 of ATM and binds directly to the kinase[92] (FIG. 2). Previous results had also implicated PP2A as a phosphatase that targets Ser1981 of ATM[93]. These recent studies provide the impetus to continue searching for additional PP2Cδ targets in the context of DNA damage, and also support the idea that PP2C function, like that of other PPP-family phosphatases, is controlled in large part by specific docking to its substrates. PP2Cδ is therefore a strong candidate for a targeted approach to cancer treatment.

Dephosphorylating the γ-H2AX tail. One of the earliest events in DNA repair is the phosphorylation of the histone 2AX tail (which yields γ-H2AX). This event generates observable, discrete nuclear foci that localize the repair machinery to the break region. One unanswered question that was addressed recently by two groups is how γ-H2AX is attenuated and removed from the site of repair. The simplest possibility is dephosphorylation by a γ-H2AX phosphatase. Keogh *et al.* began addressing this possibility by examining deletion strains for the nine nuclearly localized protein Ser/Thr phosphatases in S. *cerevisiae*[94]. Only deletion of Pph3 (PP4 in mammals) caused an accumulation of γ-H2AX. Mass spectrometry of TAP-tagged Pph3 complexes identified Psy4 and Psy2, the yeast orthologues of the R2 and R3 subunits of human PP4, as stoichiometric binding partners, and deletion of either also increased γ-H2AX levels.

Interestingly, γ-H2AX is lost from the region surrounding DNA damage independently of the PP4 complex, which indicates that γ-H2AX is dephosphorylated after displacement from chromatin. Although this same PP4 complex exists in mammals, it seems that in mammals a PP2A complex dephosphorylates γ-H2AX directly on chromatin[95]. Human γ-H2AX is also protected from dephosphorylation by its docking partner mediator of DNA-damage checkpoint-1 (MDC1), of which no orthologue exists in yeast. This illustrates an important difference between humans and budding yeast, which might account for a different dephosphorylation event. PP2A is also a key regulator of other DNA-repair proteins in humans, including ATM and DNA-PK[93,96].

Gene transcription and RNA processing
Phosphorylation of the transcription and RNA-processing machinery is well established as a means of modulating the process of yielding mature mRNA. A considerable volume of data has established that PP1 interacts with chromatin at distinct sites through several chromatin-remodelling and transcriptional regulatory complexes (for recent reviews, see REFS 77,97). We will therefore

Base-excision repair
(BER). The main DNA-repair pathway that is responsible for the repair of apurinic and apyrimidinic (AP) sites in DNA. BER is catalysed in four consecutive steps: a DNA glycosylase removes the damaged base; an AP endonuclease processes the abasic site; a DNA polymerase inserts the new nucleotide(s); and DNA ligase rejoins the DNA strand.
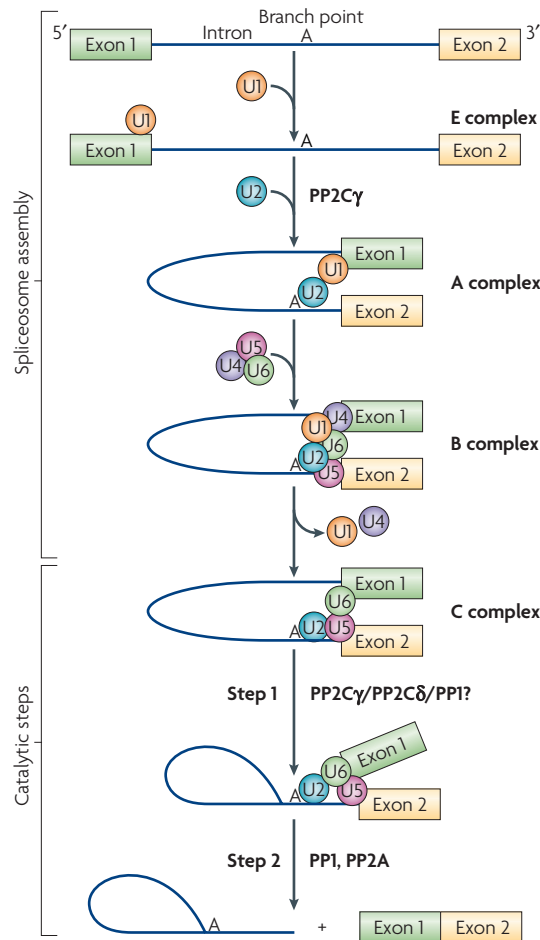
Figure 3 | **Nuclear protein phosphatases in spliceosome assembly and catalysis.** This simplified overview depicts the sequential assembly of the active spliceosome (C complex), the recruitment of small nuclear ribonucleoprotein particles (snRNPs; U1, U2, U4–6) and other splicing factors to the nascent RNA, and the two catalytic steps involved in pre-mRNA splicing. PP2Cγ activity has been shown to be required during the early steps of spliceosome assembly, specifically at the conversion of the pre-spliceosomal E complex to the A complex. Recent work has shown that PP1 and PP2A have essential (although possibly redundant) roles in the second step of catalysis[76], whereas the phosphatase activity required for the first step remains unclear. Conflicting results either support or rule out a role for PP1 at this step, and PP2Cγ and PP2Cδ (also known as WIP1) are also strong candidates. A, adenosine.

focus on the role of protein phosphatases in the control of Pol II and pre-mRNA-splicing events.

RNA processing. RNA splicing is an essential, precisely regulated post-transcriptional process that occurs before mRNA translation. After a gene is transcribed into a pre-mRNA, specific exonic sequences are retained and intronic regions are removed by a macromolecular complex known as the spliceosome. Exons can also be targeted for removal in different combinations, in a process referred to as alternative splicing, to create a diverse array of mRNAs from a single pre-mRNA.

The phosphorylation and dephosphorylation of key substrates has been shown to be essential both for assembly of the spliceosome and for the catalytic steps of RNA splicing, yet it is still unclear exactly which phosphatases are involved and at which stage(s) they function. PP2Cγ has been shown to be recruited at an early step and its activity is required for spliceosome assembly[63] (FIG. 3), whereas the inhibition of PP1 and PP2A has no effect on this process[98]. However, there are conflicting data for the roles of PP1 and PP2A in the regulation of the two catalytic steps of pre-mRNA splicing, and these have been attributed to limitations of the available phosphatase inhibitors[98,99]. Recently, Shi *et al.*[76] presented evidence that indicates that PP1 and PP2A have essential but redundant roles in splicing, and this might explain conflicts among earlier results. Also, they showed that the activities of these phosphatases are primarily required for the second catalytic step of splicing (FIG. 3), and they identified potential spliceosomal substrates. Mechanisms for targeting these phosphatases to the spliceosome remain to be defined, although at least two putative PP1-targeting subunits, PSF and NIPP1, have been shown to associate with the spliceosome and might function in the recruitment of this phosphatase[99,100]. PP2Cγ remains a possible candidate for the regulation of the first catalytic step of splicing, as does PP2Cδ, another PP2C-family member that has been found in the spliceosome-associated CDC5L complex[101].

Transcriptional control by Pol II. The transcription of protein-encoding genes in eukaryotes is carried out by Pol II. During a single round of transcription, the CTD of the largest subunit of Pol II recruits additional factors that are involved in mRNA maturation, including capping enzymes, 5′-processing factors, several splicing factors and the 3′-polyadenylation machinery. The recruitment of these processing factors depends on the phosphorylation state of the CTD of Pol II[5,15]. Pol II is recruited to the pre-initiation complex with a hypophosphorylated CTD, which is then hyperphosphorylated upon docking (FIG. 4). During elongation, additional phosphorylation of the CTD occurs. The CTD, which is composed of a heptapeptide repeat with up to 52 copies in mouse and human, gets phosphorylated on Ser2 and Ser5 of this 7-amino-acid repeat. The CTD is then dephosphorylated at transcription termination, an action that is necessary for its recruitment to a new pre-initiation complex[15].

The first phosphatase shown to dephosphorylate the CTD was FCP1, a highly conserved eukaryotic enzyme that is essential in budding and fission yeast. The N terminus of FCP1 contains the catalytic signature DXDXT/V of the Asp-based phosphatase group, whereas its C terminus has a BRCT repeat that docks FCP1 to the phosphorylated CTD[15]. FCP1 activity is also stimulated by the Pol II-associated protein RAP74. A yeast *fcp1*-mutant experiment indicated that the enzyme targets Ser2 of the CTD repeat[102]. This prompted a search for the Ser5 phosphatase, leading to the discovery of the FCP1-related enzymes SCP1–3, which lack the BRCT domain, but like FCP1 are activated by RAP74 (REF. 17). Four other
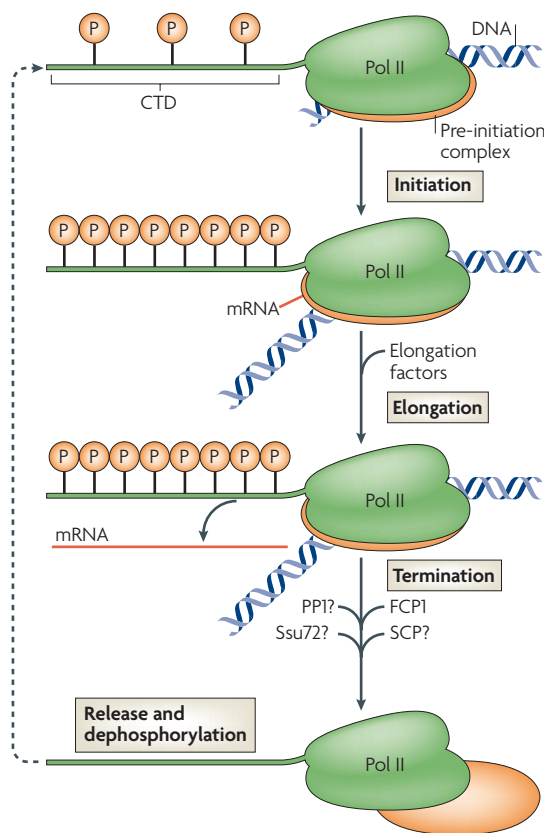
Figure 4 | The phosphorylation state of the C-terminal domain of RNA polymerase II controls transcript maturation. Transcription from RNA polymerase II (Pol II) involves docking to a pre-initiation complex and phosphorylation of the largest subunit of Pol II on its C-terminal domain (CTD). During mRNA biogenesis, specific mRNA-processing factors are recruited depending on the phosphorylation pattern of the CTD, which changes during the transcription cycle. At termination, protein phosphatases function on the CTD, allowing Pol II to be recruited to another pre-initiation complex. The phosphatase FCP1 (TFIIF-associating CTD phosphatase-1) is essential for this dephosphorylation event. Other data indicate a role for PP1, Ssu72 and the small CTD phosphatases (SCPs), although a precise role has not yet been defined for these phosphatases. P, phosphate.

putative CTD phosphatases have been identified in the human genome[5] (TABLE 1). SCPs seem to have a role in neuronal gene silencing[16] and, recently, new substrates that function in the TGFβ and BMP pathways have been identified (see below). The SCPs indeed seem to target the Ser5 residue of the CTD[17], and it was shown recently using highly purified FCP1 that this enzyme also preferentially dephosphorylates Ser5 of the CTD *in vitro*[103]. The crystal structure of SCP1 in complex with singly and doubly phosphorylated CTD peptides, in combination with kinetic data, beautifully disclose why the enzyme preferentially dephosphorylates Ser5 (REF. 5).

The Ssu72 protein was identified in yeast as an essential factor that functions during all phases of transcription[15]. Sequence analysis of Ssu72 revealed the conserved PTP catalytic signature (CX_5R), and depletion of

Ssu72 *in vivo* increased Ser5 phosphorylation of CTD. Other searches for a CTD phosphatase have indicated a role for PP1, which is consistent with data showing that okadaic acid treatment increases CTD phosphorylation[77]. Additional work is needed to clearly define the CTD phosphatases and their individual roles in transcriptional control.

TGFβ and BMP signalling

TGFβ has a role in cell proliferation, differentiation and embryonic development in a range of metazoan organisms from worms to humans[104]. When TGFβ binds its target receptor Ser/Thr kinases at the cell membrane, it promotes receptor activation through phosphorylation, ultimately resulting in the recruitment and phosphorylation of the cytosolic target SMAD proteins on their C-terminal SXS sequences. This allows the SMADs, including SMAD2 and SMAD3, to interact with the phosphoserine-binding MAD homology-2 (MH2) domain of SMAD4, which results in the translocation of this complex to the nucleus, allowing the regulation of ligand-responsive gene expression[104] (FIG. 5). The SMADs are also phosphorylated in their N termini and linker regions by other protein kinases[104–106].

PP2C, SCPs and SMAD-protein dephosphorylation. Previous studies had indicated that dephosphorylation of SMAD2 and SMAD3 was necessary to terminate TGFβ signalling. To identify the phosphatase responsible, Lin *et al.* individually expressed 39 different catalytic subunits of phosphatases (primarily protein phosphatases) in cells and assayed the dephosphorylation of the C termini of SMAD2 and SMAD3 (REF. 105). Only PP2Cα was capable of this dephosphorylation event, and this was supported by *in vitro* experiments showing that recombinant PP2Cα could dephosphorylate SMAD2 directly. This group next went on to show that PP2Cα could physically bind SMAD2 and SMAD3, and remarkably, the phosphatase had a higher affinity for phosphorylated SMAD2 and SMAD3 compared with the non-phosphorylated forms.

Additional experiments revealed that PP2Cα was exclusive to the nucleus, that non-phosphorylated SMAD2 and SMAD3 were present in the cytosol and that TGFβ stimulation of the pathway promoted the interaction of PP2Cα and phospho-SMAD2 and -SMAD3 in the nucleus. Upon dephosphorylation of the SXS sequence, SMAD2 and SMAD3 lost the ability to interact with SMAD4 in the nucleus and shuttled back to the cytosol (FIG. 5). PP2Cα is expressed at different levels in various cells types, and this could therefore cause a differential response to TGFβ among tissues. It has also been suggested that if the PP2Cα activity or localization is not regulated, nuclear SMADs could be continuously dephosphorylated and exported from the nucleus. Once the TGFβ receptors are inactivated, this would cause a rapid shutdown of the signalling pathway. Other work has shown a key role for the PP1-docking protein Sara as the scaffold that is responsible for presenting PP1 to the TGFβ receptor (FIG. 5) to promote dephosphorylation and signal attenuation[107].
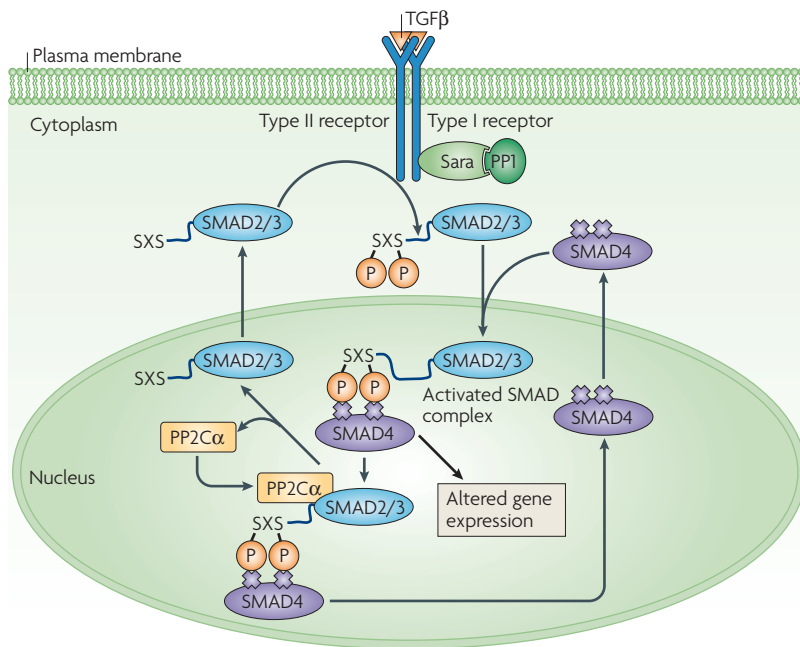
Figure 5 | **PP2Cα attenuates TGFβ signalling in the nucleus.** The binding of transforming growth factor-β (TGFβ) to pairs of target type I and II membrane receptor Ser and Thr kinases activates the receptor and allows phosphorylation of SMADs (in this case SMAD2 and SMAD3) on their C-terminal SXS motifs. This generates a binding motif for the MAD homology-2 (MH2) domain of SMAD4. Once associated, SMAD2, SMAD3 and SMAD4 shuttle into the nucleus where they associate with various DNA-binding cofactors and transcriptional co-activators or co-repressors that regulate the expression of several hundred genes. In the nucleus, the signal is terminated by a dephosphorylation event that is controlled by PP2Cα. Dephosphorylation of SMAD2 and SMAD3 results in decreased affinity for SMAD4, and both proteins exit the nucleus to complete the cycle. The docking of PP1 to the TGFβ receptor through Sara causes receptor dephosphorylation and signal attenuation. Not shown is the (de)phosphorylation of the N-terminal and linker regions of SMAD2 and SMAD3, which are targeted by the small C-terminal domain phosphatases (SCPs) (see main text). P, phosphate.

Two groups have now studied the dephosphorylation of SMAD2 and SMAD3 in the linker and N-terminal regions using a similar functional genomics approach[108,109]. This combined work identified SCP1–3 (but not FCP1) as specific linker phosphatases and showed that SCP1 and SCP2 function to enhance TGFβ signalling.

Dephosphorylation of BMP-activated SMADs. The BMP proteins belong to the TGFβ superfamily and signal in a similar manner to TGFβ (FIG. 5) by receptor activation and subsequent phosphorylation of SMADs on their C-terminal SXS motifs and by binding to SMAD4 to enter the nucleus. However, they target SMAD1, SMAD5 and SMAD8. An RNAi screen for the SMAD1 phosphatase in *D. melanogaster* S2 cells identified the pyruvate dehydrogenase phosphatase (PDP) as the dephosphorylating enzyme, and this observation was extended to human cells[110]. In addition, PDP was shown to have no effect on TGFβ-activated SMAD2 and SMAD3. Knockaert *et al.* subsequently reported the dephosphorylation of the C terminus of SMAD1 by SCP1–3 (REF. 111).

More recently, the Feng group reported that PP2Cα could dephosphorylate all C-terminal SXS motifs, and,

in their hands, overexpression of the PDPs or SCPs had no effect on SXS phosphorylation of SMAD1 (REF. 112). They suggested that additional cofactors, which are not present at sufficient levels to accommodate overexpression studies, might account for the discrepancy between the works. Clearly, additional studies are needed here to determine whether each of these enzymes functions as a SMAD phosphatase and perhaps provides inputs from different signalling pathways in the cell.

Future directions
The field of protein phosphatase research has entered a new era for several reasons. First, because of what is probably a nearly complete inventory of catalytic subunits in several organisms, it is now possible to do large-scale or functional genomics screens for the role of phosphatases in specific biological processes. Second, as more phosphatase-targeting subunits are identified and characterized, it is increasingly clear that they have a fundamental role in phosphatase function and that a comprehensive catalogue of these proteins is crucial to our understanding of the varied intracellular functions of their associated catalytic subunits.

Targeting these regulatory subunits by various means or disrupting their interaction with the catalytic subunit might also represent a feasible means to treat specific diseases. In light of this and the linking of certain phosphatase catalytic subunits to disease genes[11], phosphatases have at last emerged as legitimate drug targets[3–5]. The potential of this idea was typified in the large-scale loss-of-function screen done by Mackeigan and colleagues[8]. Even without a comprehensive list of phosphatases and regulatory subunits, this group found that a surprising 32% of the phosphatases screened promoted cell survival, whereas 10 protein phosphatases were classified as 'cell-death phosphatases' (that is, the reduction of their cellular levels caused resistance to apoptosis-inducing agents), and can therefore be considered putative tumour suppressors. In addition to the uncharacterized PTPs and growing Asp-based phosphatase family (TABLE 1), more potential drug targets are likely to emerge as additional regulatory subunits and complexes are defined.

Mass-spectrometry-based proteomics has become a powerful tool to identify the components of multiprotein complexes, and more recently, several techniques have exploited the use of heavy isotope tags to compare and quantitate relative protein levels under different biological conditions (for reviews, see REFS 38,113). An example was the use of SILAC-based mass spectrometry to rapidly identify PP1-interacting proteins in nuclear lysates and to distinguish between preferential binding of these proteins to either the α- or γ-isoform of PP1 (REF. 34). Proteomics approaches were also used to identify the specific PP2A complex that is involved in the regulation of chromosome segregation[78,79] and the newly identified PP4 complexes[44]. Clearly, mass-spectrometry-based approaches and large-scale functional genomics will be major driving forces in this new era in which protein phosphatases will sit more prominently on centre stage with their protein kinase counterparts.

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DATABASES
The following terms in this article are linked online to:
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FCP1 | IGBP1 | NIPP1 | p99 | SMAD1 | SMAD2 | SMAD3 | SMAD4 | SMAD5

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Prokaryotic Protein Phosphatase Database: http://vigen.biochem.vt.edu/p3d/p3d.htm
Protein phosphatase-1 (PP1) binding proteins: http://pp1signature.pasteur.fr/index.html
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