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Protein Serine/Threonine Phosphatases: Keys to Unlocking Regulators and Substrates

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Abstract

Protein serine/threonine phosphatases (PPPs) are ancient enzymes, with distinct types conserved across eukaryotic evolution. PPPs are segregated into types primarily on the basis of the unique interactions of PPP catalytic subunits with regulatory proteins. The resulting holoenzymes dock substrates distal to the active site to enhance specificity. This review focuses on the subunit and substrate interactions for PPP that depend on short linear motifs. Insights about these motifs from structures of holoenzymes open new opportunities for computational biology approaches to elucidate PPP networks. There is an expanding knowledge base of posttranslational modifications of PPP catalytic and regulatory subunits, as well as of their substrates, including phosphorylation, acetylation, and ubiquitination. Cross talk between these posttranslational modifications creates PPP-based signaling. Knowledge of PPP complexes, signaling clusters, as well as how PPPs communicate with each other in response to cellular signals should unlock the doors to PPP networks and signaling "clouds" that orchestrate and coordinate different aspects of cell physiology.

Contents

1. INTRODUCTION

1.1. Protein Phosphorylation and Dephosphorylation in Cell Signaling

Reversible protein phosphorylation has been established as a major control mechanism for virtually all aspects of eukaryotic cell physiology. This mechanism was first discovered in the early 1950s in studies of the interconversion of glycogen phosphorylase from an active *a* form to an inactive *b* form (1, 2). To date, glycogen phosphorylase remains one of the simplest examples of phosphoregulation with only one serine (Ser) phosphorylated by one protein kinase, namely phosphorylase *b* kinase. Now six decades since these early studies, researchers understand that most proteins are phosphorylated by multiple kinases at several sites, which allows for the integration of a wide range of physiological signals and also generates different kinetic or functional outcomes (3). Metabolic labeling and phosphoamino acid analysis first estimated that phosphotyrosines (pTyr) accounted for <1%, with the remainder of the protein-bound phosphate in phosphothreonine (pThr) and phosphoserine (pSer). The tenfold relative increase in pTyr content that occurred in cells transformed by Rous sarcoma virus (4) drew attention to the importance of this modification in cancer biology (5). Decades later, the more sophisticated experimental approach of stable isotope labeling of amino acids in cell culture (SILAC) combined with mass spectrometry of approximately 2,000 phosphoproteins (6) yielded a distribution of 1.8% pTyr-containing sites,

with pThr and pSer accounting for 98.2% of the total protein-bound phosphate. The PhosphoSitePlus database based mostly on shotgun mass spectrometry documents nearly 170,000 pSer-, 70,000 pThr-, and 44,000 pTyr-containing sites. These different data highlight that the vast majority of phosphorylations on eukaryotic proteins occur on Ser and Thr. This is consistent with the fact that more than 80% of the more than 500 protein kinases encoded in the human genome catalyze phosphorylation on Ser and Thr (7). This review focuses on the major protein phosphatases that catalyze dephosphorylation of pSer and pThr residues in controlling eukaryotic cell physiology.

The earliest example of reversible phosphorylation suggested that phosphorylase became activated following its phosphorylation. Dependency on allosteric activation by metabolites such as glucose and AMP was greatly reduced but was restored following dephosphorylation (8). This set the foundation for thinking that phosphorylation at Ser and Thr elicits a conformation change that alters enzyme activity or protein function. Indeed, there are many examples of protein phosphorylation that activate cellular enzymes; among these are many protein kinases: Raf, MEK, MAPK, AKT, AMPK, TGFβR, and IKK. However, what is frequently not appreciated is that Ser/Thr phosphorylation also inactivates many enzymes, such as glycogen synthase (9), AcCoA carboxylase (10), HMG-CoA reductase (11), src family kinases (12), and CDKs (13). These enzymes are reactivated by their dephosphorylation, most by protein Ser/Thr phosphatases (PPPs). Thus, protein phosphatases can produce changes in the conformation of substrate proteins that switch them either on or off.

Phosphorylation of Ser/Thr residues also generates docking sites for a variety of phosphodependent protein interaction domains or modules (14, 15). Domains, such as 14-3-3, FHA, BRCT, WW, WD40, and Polo-Box recognize sequences that contain pSer or pThr residues. This phosphorylation-dependent binding provides for the regulated recruitment of protein partners and the conditional assembly of multiprotein signaling complexes. Binding of these pSer/pThr recognition domains potentially protects phosphorylation sites from being efficiently dephosphorylated by PPPs. As such, the persistence or life span of individual pSer/pThr sites probably reflects competition between pSer/pThr-binding partners and the protein phosphatases that dephosphorylate these specific sites.

Protein phosphorylation is highly dynamic: Experimental measurements of half-lives for some phosphosites are in the order of seconds (16, 17). High turnover (cycling) of phosphosites, though seemingly wasteful in terms of ATP utilization, offers some advantages, specifically functioning as a noise filter and enabling versatile tuning of cellular signals (18). Yet other mathematical analyses suggest that rapid kinase/phosphatase cycles increase cellular sensitivity or responsiveness to physiological stimuli and control other aspects of cell signaling, including time course or dynamics, cross talk, and signal amplification. In addition, high phosphatase activity allows for more accurate kinetic proofreading of phosphorylation events, rather than functioning simply as a shut-off mechanism. In any signaling pathway, the concerted efforts of all the negative regulators are required to achieve effective termination of a signal response (19). Last but not least, some mathematical models of kinase signaling suggest that phosphatases may have a more dominant role in determining the speed and duration of a signal response, whereas kinases may be more prominent regulators of the amplitude of a physiological response (20). Gelens et al. (21) have expanded this concept in a review article. They argue that kinases and phosphatases often cooperate to dictate the amplitude and timing of signal transduction as well as the localization and directionality of signaling during cellular events such as mitosis. These analyses emphasize that, although protein phosphatases are commonly viewed as simply negative regulators or "erasers" that turn off cell signaling, they also play important roles in origination, transduction, and transmission of cellular signals.

1.2. Distinct Families of PPPs

Early biochemical studies separated mammalian PPPs into two major groups, namely type-1 or type-2, based on their in vitro substrate specificity with selected phosphoprotein substrates and their sensitivity to endogenous protein inhibitors (22). This classification scheme had a dominant and long-lasting impact. However, molecular cloning later exposed multiple types of PPPs, and further functional analyses defined three broad families of Ser/Thr phosphatases—PPP, PPM, and HAD—that are distinguished by their primary structures and their mechanisms of catalysis (23). Together, the PPP family and the protein phosphatase Mg^{2+}-dependent (PPM) family are encoded by a total of ~40 mammalian genes, which is at least 10 times fewer than the number of genes that encode mammalian protein Ser/Thr kinases. This imbalance between kinases and phosphatases prompted the popular speculation that phosphatases must be much less specific in their recognition of substrates and likely catalyze the dephosphorylation of many different phosphoproteins that are substrates of the more selective and numerous protein kinases. However, as described in reviews over the past 15 years (24–26), there is compelling evidence that PPPs rarely, if ever, exist as free catalytic subunits but instead are incorporated into multisubunit complexes (holoenzymes) in which the catalytic subunit is bound by one or more regulatory subunits that dictate its catalytic activity and determine substrate specificity, subcellular localization, and regulation. To date, researchers have identified more than 200 regulators for protein phosphatase 1 (PP1) and more than a dozen genes and isoforms for B regulatory subunits of PP2A, the major PPPs in most mammalian tissues. Thus, the number of PPP complexes in eukaryotic cells approximately equals the number of Ser/Thr kinases.

This situation, however, should not be extrapolated to suggest there is one kinase and one phosphatase to regulate any single phosphoprotein (or a single phosphosite). The cellular repertoire of phosphoproteins has gone through fantastic expansion, from 6,000 unique phosphorylation sites in the human phosphoproteome in 2006 (6) to more than 38,000 by 2014 (27), and a significant fraction of phosphoproteins are modified at multiple Ser/Thr sites. The number of phosphosites is likely to increase even further as the phosphoproteome is interrogated following physiological perturbations of cells or as phosphoprotein profiles are completed in different tissues and cell types. Given this tremendous number of phosphorylation sites, every kinase and every phosphatase has to be expected to have numerous substrates. The defining example of phosphorylase regulation by a single site of phosphorylation has proved to be more an exception than the rule. The presence of multiple phosphorylation sites in proteins enables more diverse and variable physiological outputs than are possible with the simple one-site model. Moreover, control of the phosphorylation state of any single Ser or Thr by multiple protein kinases and phosphatases allows cells to integrate a range of inputs to control protein function.

The type-1 and type-2 classification scheme from the 1980s assigned one type of PPP as PP2C, but this turned out to be the founding member of the separate PPM family, with sequences distinct from those of PPPs. The architecture of PPM catalytic domains [44 PPM structures determined by X-ray crystallography in the Protein Data Bank (PDB)] is characterized by the presence of a pair of active site divalent metal ions (Mg^{2+} or Mn^{2+}), and the proposed catalytic mechanism involving metal bound water is similar to those of PPPs (28). More recent kinetic and structural evidence shows these PPM phosphatases actually use three metal ions at their active sites; the third ion binds with relatively low affinity, consistent with the dependence on millimolar levels of added Mg^{2+} for optimal activity (29, 30). PPMs are widely expressed across prokaryotic and eukaryotic species, including mycobacteria and plants, and compared with the 16 mammalian PPM genes, it is astonishing that plants possess 80 to 90 PPM genes. Thus, PPMs are a major family of Ser/Thr phosphatases in plants where they transduce hormonal signals as a subunit of the abscisic acid

receptor (a ligand-inactivated phosphatase) and modulate a variety of stress responses (reviewed in 31). Despite appearing in early assays as a comparatively small fraction of the total pSer/pThr phosphatase activity (probably owing to the choice of substrates then used), PPMs have prominent and important roles in growth regulation and stress signaling in mammalian cells (reviewed in 32).

Haloacid dehydrogenases (HADs) represent the third family of Ser/Thr phosphatases and exhibit relatively low activity (reviewed in 33). Unlike PPPs and PPMs, HADs, such as FCP/SCP, are not metalloenzymes but have a conserved DXDX(T/V) sequence in their catalytic site. In addition to their low substrate turnover, HADs display extremely narrow substrate specificity, compared with PPPs or PPMs, limited to dephosphorylation of selected pSer residues located within the C-terminal tail of RNA polymerase II (34). HADs and PPMs will not be discussed further, and this review focuses on PPPs.

Cloning of PPP catalytic subunits eventually revealed seven distinct types in the human genome, referred to as PPP1 to PPP7. A number of different names persist in the current literature, but in this review we use PP1, PP2A, PP3 (also known as PP2B or calcineurin), PP4, PP5, PP6, and PP7. PP7 is also known as PPEF (protein phosphatase with EF-hand domains) (35) or RdgC-like phosphatase, following identification of the *Drosophila RdgC* gene, whose loss of function leads to retinal degeneration. In this review, we focus our discussion on three areas: (*a*) the molecular determinants that dictate subunit and substrate recognition; (*b*) the mechanisms that control PPP functions, specifically cross talk between various posttranslational modifications (PTMs); and (*c*) the manner in which PPPs communicate or cooperate to control signaling networks. Our emphasis on PP1 and PP2A results primarily from two factors: These enzymes are the major Ser/Thr phosphatases in various eukaryotic cells, and owing to their early discovery and extensive studies, there is a wealth of information about these PPPs compared with other PPPs that allows us to glean principles that may influence and benefit future research on the other PPPs.

1.3. Evolutionary Conservation and Segregation of PPPs

PPPs are phosphomonoesterases (EC3.1.3), and genes with related sequences appear in all eukaryotes as well as in bacteria and archaebacteria, highlighting their remarkable conservation through evolution (36, 37). PPPs are essential for cell viability, particularly in simple eukaryotes that possess only a single gene encoding any given PPP (38). It is presumed that lethality in animal cells due to loss of PPP is avoided by the presence of multiple PPP isoforms that can fulfill overlapping functions. Supporting their critical roles in eukaryotic biology, PPPs are subject to inhibition by a variety of xenobiotics, such as polyketides (e.g., okadaic acid, calyculin A) and cyclic peptides (e.g., microcystin, nodularin) that bind in PPP active sites. By abrogating PPP functions, these compounds are cytotoxic (39). Since their discovery nearly 25 years ago, these toxins have become extremely valuable tools for analyzing the physiological functions of PPPs and greatly enabled the progress of phosphatase research in the years before molecular cloning of multiple PPPs (40, 41). They are useful tools because some are cell permeable and they do not inhibit PPMs or HADs. Multiple PPPs that are sensitive to these toxins, namely PP1, PP2A, PP4, PP5, and PP6, are common among eukaryotes. Therefore, we urge caution in attributing the effects of any toxins reported in the literature to the inhibition of a single type of PPP. Although insensitive to PPP toxins, PP3 is inhibited by two other natural products, namely cyclosporin and FK506, that have been used as immunosuppressive drugs to counteract graft-versus-host disease following organ transplantation. The success of these drugs raises the possibility that inhibitors targeting individual PPPs may be developed to treat other human diseases (42).

PPPs are among the most evolutionarily conserved of all enzymes. Although stringently segregated into separate types, each PPP type serves distinct, species-specific functions. Human type-2

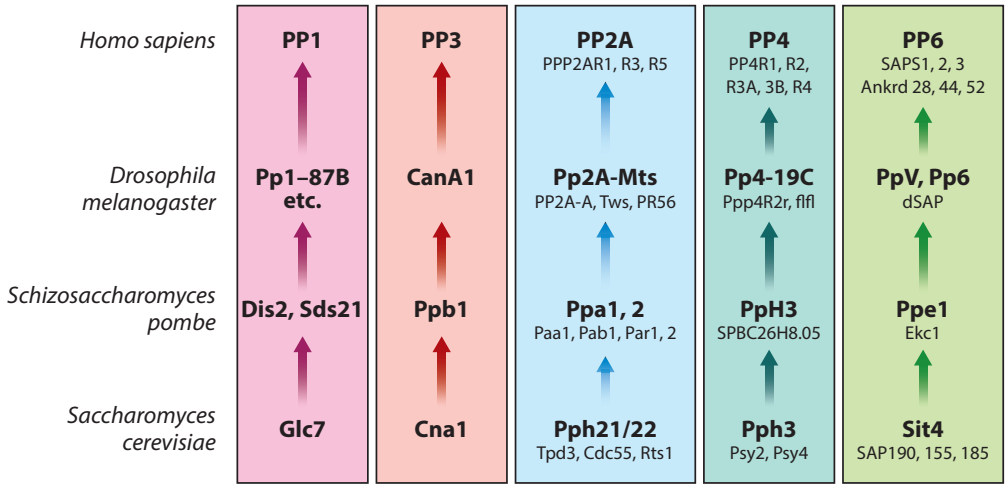

Figure 1

Evolutionary segregation of protein serine/threonine phosphatases (PPPs). The scheme shows the conservation of multisubunit PPPs during evolution from fungi to human, with names for enzymes of different organisms. Experiments have exchanged genes between species to show that the genes of a single PPP type, represented in different colored boxes, can functionally substitute for another of the same type but not for genes of another PPP type. This segregation is attributed to the association of these catalytic subunits with regulatory subunits specific for each PPP type, indicated for the various species using smaller font. Although the holoenzymes are conserved between species, their substrates, and thus the physiological functions of each PPP type, are not the same in various species.

PPPs, namely PP2A, PP4, and PP6, are more closely related in sequence to one another than they are to the other PPPs. These phosphatases are preserved as separate genes in *Saccharomyces cerevisiae* (PPH21, PPH3, and SIT4), *Schizosaccharomyces pombe* (ppa2, pph3, and ppe1), *Caenorhabditis elegans* (LET-92, PPH-4.1, and PPH-6), and *Drosophila melanogaster* (mts, Pp4-19C, and PpV) (**Figure 1**). Knockdown of these individual type-2 PPPs by RNAi in *C. elegans* results in different phenotypes (43–45), illustrating the concept that each PPP fulfills separate functions in a given species. Mutations in either Sit4 or Ppe1 (46) are complemented by human PP6, establishing the functional equivalency of type-6 PPP from divergent species. By contrast, neither PP4 nor PP5 rescued the *S. cerevisiae* Sit4-102 mutation, thereby reinforcing specificity of the Sit4 homolog PP6. The ability of human PP6 regulatory Sit4-associated protein subunits (SAPSs) to associate with yeast Sit4 and to functionally replace the yeast Sit4-associated proteins (SAPs) suggests that these regulatory proteins have an equally critical role in the function of PP6 in different species (47). Therefore, human PPPs can substitute for their homologs in other species, but this applies only to the single type of PPP and not to other PPPs. This segregation of PPP types is attributed to the specific binding to regulatory subunits of each PPP type. Complementary structures in catalytic and regulatory subunits mediate the assembly of distinctive multisubunit holoenzymes, and these features are conserved across evolution.

Although the PP6 phosphatases Sit4, Ppe1, and PpV are functionally equivalent and interchangeable in terms of genetic complementation, there is not a conserved function they fulfill in multiple species, in terms of their phosphoprotein substrates or control of cellular or physiological processes. This implies that substrate recognition by particular holoenzymes is mutable across species, even though holoenzyme assembly is not. The PP6 in budding yeast, called Sit4, regulates transcription of G1 cyclins (48), presumably by dephosphorylation of transcription factors that regulate cyclin expression, but the PP6 holoenzyme Ppe1::Ekc1 in fission yeast dephosphorylates the Mis12 complex at kinetochores to regulate chromosome segregation (49). In *Drosophila*, the

PP6 homolog PpV is located in fat droplets, where it dephosphorylates AMPK to regulate lipid metabolism (50). In human cells, PP6 and its SAPSs bind to DNA-PK to enable non-homologous end joining of double-strand breaks in DNA following exposure to ionizing radiation (51, 52). In HeLa cells during mitosis, PP6 dephosphorylates and inactivates Aurora A kinase (53) and dephosphorylates the condensin complex (54). In human epithelial cells, PP6 is concentrated at adherens junctions and associates with and dephosphorylates E-cadherin to control its exposure on the cell surface (55). Thus, separate PPP and dedicated regulatory subunits have been preserved together as holoenzymes during evolution, but in each species, PPP can engage different substrates and therefore fulfill different assignments.

This principle that substrates identified in one species do not necessarily predict the function of a PPP in another species applies to PP7 as well. For example, *RdgC* is a member of the PP7 family first identified in *Drosophila*, where its loss of function results in retinal degeneration (56). Subsequent studies in fruit flies showed that *RdgC* dephosphorylates rhodopsin (57). The presence of a Ca^{2+}-binding EF-hand domain identified *Rdgc* in a distinct PP7 family that is expressed in plants and animals (see 35). Mammals contain two PP7 isoforms, PPEF1 and PPEF2, primarily expressed in sensory neurons, although lower levels are seen in other cells. The loss of function of both PPEF1 and PPEF2 in mice does not result in retinal degeneration, and rhodopsin dephosphorylation is not impaired in mutant mice (58). Plant PP7 is primarily nuclear and dephosphorylates the zinc finger protein HRB1 to regulate stomatal opening (59). These data reinforce the idea that PP7 serves quite distinct functions in flies, mammals, and plants and highlight the challenges of defining PPP functions across species.

There are functional differences even between isoforms of a single PPP type. Human PP1 catalytic subunit is encoded by *PPP1CA*, *PPP1CB*, and *PPP1CC*, and these three genes share more than 80% sequence identity with *GLC7*, the single PP1 gene in budding yeast. Individual human PP1 isoforms effectively restore cell viability in yeast that lack Glc7 function (60). More detailed analysis of specific Glc7-regulated events in yeast, however, revealed that none of the human PP1 isoforms completely matched the ability of Glc7 to regulate glycogen synthesis, gene regulation, or cell division in yeast. These data suggest some functions of individual PPP isoforms have diversified during evolution.

Some PPPs were apparently lost during evolution because they are expressed in fungi but not in higher organisms. PPZ is a member of the PPP family whose C-terminal catalytic domain shares 60% sequence identity with mammalian PP1 (61). Yeast PPZ1 and PPZ2 isoforms regulate salt tolerance and oxidative stress. PPZ binds some yeast PP1 regulatory proteins, such as Glc8 (62) and Ypi1, orthologs of mammalian inhibitor-2 (I-2) and inhibitor-3 (I-3), respectively (63). However, these proteins did not inhibit PPZ activity. Recombinant PPZ1 does not dephosphorylate phosphorylase but is inhibited by okadaic acid and microcystin (64). Recent X-ray crystallography of the PPZ catalytic domain established that it binds microcystin in an identical manner to PP1. Moreover, PPZ possessed an RVxF pocket, with 90% identity to PP1, thus explaining how it bound some but not all PP1 regulators (65). However, this pocket was not utilized by the yeast PPZ-specific inhibitor HAL3, which inhibited PPZ activity through an alpha helix (66) similar to PP1 inhibition by I-2 (67). PPQ (also known as SAL6) is another PPP that is expressed solely in fungi (68). We speculate that the actions of PPZ and PPQ became dispensable in higher eukaryotes, leading to their loss from genomes.

1.4. Active Site Metal Ions in PPPs

Years ago, Mn^{2+} was added routinely to enhance protein phosphatase activity, even though it was unclear whether the metal ion bound to the enzyme. Vincent & Averill (69) then pointed out the

sequence similarity between PPP and purple acid phosphatases, specifically in residues that create a bimetallic (Fe::Zn) active site. In these enzymes, a water molecule bridges the metal ions and acts as nucleophile in the direct hydrolysis of pSer/pThr residues in substrates (70). The similarity of PPP to purple acid phosphatase was confirmed by analyzing iron and zinc ions in PPPs (i.e., calcineurin and PP2Ac) purified from cells or animal tissues (71, 72) and in one recombinant PP1 structure (73). There are now many PPP three-dimensional structures in the PDB: 31 PP1, 25 PP2A, 15 PP3, and 16 PP5 structures. Yet, structures for PP4, PP6, and PP7 remain unsolved. Recombinant PPPs expressed in bacteria or insect cells possess two Mn^{2+} ions at their active site, in contrast to the Fe^{2+}::Zn^{2+} pair at the catalytic center of the native enzymes from tissue sources. The presence of the native bimetallic center in PP1 has a significant impact on its biochemical properties, such as limited ability to hydrolyze small-molecule substrates, such as *p*-nitrophenyl phosphate or phosphopeptides, and sensitivity to endogenous protein inhibitors (74). These differences in PP1 properties suggest some altered protein conformation depending on the active site metal ions, but no differences between native and recombinant enzymes have been detected since the first solution of the PP1 structure by X-ray crystallography nearly 20 years ago (75). Furthermore, the X-ray crystallography structure of recombinant PP3 expressed in bacteria containing two Mn^{2+} ions (76), compared with PP3 purified from bovine brain that contains Fe^{2+} and Zn^{2+} (71), showed no differences in protein conformation. Thus, there is little evidence that the identity of the active site metals (Fe::Zn or Mn::Mn) alters the three-dimensional structure of PPPs, even though there are measurable differences in enzyme properties. It is curious that some recombinant PP1 expressed in insect (Sf9) cells displays biochemical properties similar to native PP1 (77). This raises the possibility that specific chaperones are required for incorporation of Fe^{2+} and Zn^{2+} into PP1 during a maturation process that results in the native conformation (78). The question of how different PPPs acquire and assemble their Fe^{2+}:Zn^{2+} catalytic centers continues to perplex investigators in the field.

The issue of PPP maturation as an active enzyme harkens back to studies in the 1970s and 1980s of the inactive PP1:1–2 complex commonly isolated from mammalian tissues and described as the ATP:Mg-dependent phosphatase, which was activated following phosphorylation of I-2 by GSK-3 and dephosphorylation of I-2 by the bound PP1 (79). This ATP:Mg-dependent activation process was preserved in the recombinant PP1:I-2 complex (80). Coexpression of PP1 with I-2 in bacteria for crystallographic study yielded a PP1:I-2 complex, but this complex contains one Mn^{2+} or no metals (67). The structure showed that I-2 binding occludes the PP1 catalytic site and displaces the metals or prevents their incorporation into PP1.

Yet, by some dynamic process, I-2 can facilitate the refolding of recombinant PP1 into a conformation that more closely resembles the enzyme isolated from mammalian tissues (81). The ability of the PP1 regulator SDS22 to recruit a Cdc48-Shp1 chaperone complex (82) and I-3 has suggested that Sds22/I-3 complex may also promote the folding of PP1 into its native conformation (83). Moreover, I-2 (Glc8 in *S. cerevisiae*), Sds22, and I-3 (Ypi1 in budding yeast) are among the oldest and most evolutionarily conserved PP1-interacting proteins, consistent with their participation in biogenesis of the PP1 catalytic subunit. In an analogous manner, PTPA is a PP2A-interacting protein that possesses peptidyl-prolyl isomerase activity. PTPA binds the C-terminal tail of PP2A (84) and possibly also PP4 and PP6 (85). It can function as an ATP-dependent chaperone to enhance PP2A activation and promote the binding of metal ions (86, 87). Whether the three-dimensional folding of other PPPs, such as PP3, PP5, and PP7, requires specialized chaperones to insert Fe^{2+} and Zn^{2+} and promote folding into the native active conformation is currently unknown.

2. PPP INTERACTIONS WITH SUBSTRATES AND SUBUNITS

2.1. Contrasting Substrate Specificity of Protein Kinases and PPPs

Substrate consensus sequences or recognition motifs for different protein Ser/Thr kinases have been deduced by alignment of primary sequences surrounding the pSer and pThr in substrates. This raised hopes that a similar approach might work for PPP, but to no avail. Phage displays and peptide arrays further refined the kinase motifs to identify which residues are required for optimal substrate phosphorylation (88). This information has led to the development of predictive algorithms (e.g., GPS3.0, SCANSITE, PHOSIDA, NetPhos3.1) that can be used to query protein sequences for potential phosphorylation sites or to predict kinases that phosphorylate specific sites. This approach is not totally foolproof because some sequences may be recognized by multiple Ser/Thr kinases, but it has led to the widespread use of synthetic peptides with sequence motifs as in vitro substrates to assay kinase activity. These peptides, often relatively short (e.g., ~6 residues) in length, exhibit much lower affinity (and higher K_M) as substrates compared with the proteins that contain these sequences. Thus, additional determinants, possibly secondary structure, and additional contact sites may enhance the phosphorylation of full-length proteins by protein kinases. By comparison, synthetic phosphopeptides generally are very poor substrates for most PPPs, and to date, no dependable consensus sequences are known for PPP substrates (89, 90). PPP activity can be reliably assayed with the nonspecific small-molecule substrate DiFMUP (6,8-difluor0-4-methylumbelliferyl phosphate) that produces a fluorescent product, allowing continuous real-time readout of activity (91).

The three-dimensional structure of PP5 was determined recently with a phosphomimetic substrate peptide fused to the PP5 catalytic domain (92). The substrate peptide represents a phosphorylation site in the PP5 substrate Cdc37 and provides the first view of how PPPs bind their substrates. The Cdc37 peptide occupies the catalytic site in a similar manner to how PP1 binds the inhibitory toxins, okadaic acid (93), microcystin (75), and tautomycin (94). PP1 catalytic sites have three channels or clefts, known as the basic groove, hydrophobic groove, and C-terminal groove, that radiate out from the bimetallic catalytic center. The Cdc37 peptide occupies the hydrophobic and C-terminal grooves (**Figure 2*a***). This structure emphasizes that, except for the residue Asn308, there is nearly complete conservation of the peptide-binding residues in the catalytic sites of PP5 family members and other PPPs. Interestingly, the mutation N308D in PP5 impairs Cdc37 dephosphorylation, highlighting the molecular basis for the intrinsic specificity of PP5, because other PPPs do not dephosphorylate Cdc37. N308D, however, has no effect on the PP5-mediated dephosphorylation of the glucocorticoid receptor. This points to flexibility or plasticity in substrate recognition, with the Cdc37 peptide sitting in conserved but spacious grooves forming water-mediated hydrogen bonds with PP5. The versatility of the water-mediated interactions means that PP5, and likely other PPPs, can accommodate substrates with widely differing side chains, explaining the difficulty of establishing substrate recognition motifs for PPPs.

Early studies of isolated catalytic subunits of PP1 and PP2A utilized dephosphorylation of Ser15 in phosphorylase, which was widely adopted as a substrate for assaying phosphatase activity. Yet, these two phosphatases showed distinct preferences for dephosphorylating pSer located in different subunits of phosphorylase *b* kinase (**Figure 2*b***). Preferential dephosphorylation of phosphorylase *b* kinase subsequently became the key criterion for distinguishing type-1 PPPs that selectively dephosphorylate the beta subunit from type-2 PPPs that preferentially dephosphorylate the alpha subunit (95). Both sites in phosphorylase *b* kinase conform to the consensus motif of protein kinase A (PKA) that has tandem basic residues (RR or KR) to the N-terminal side of the

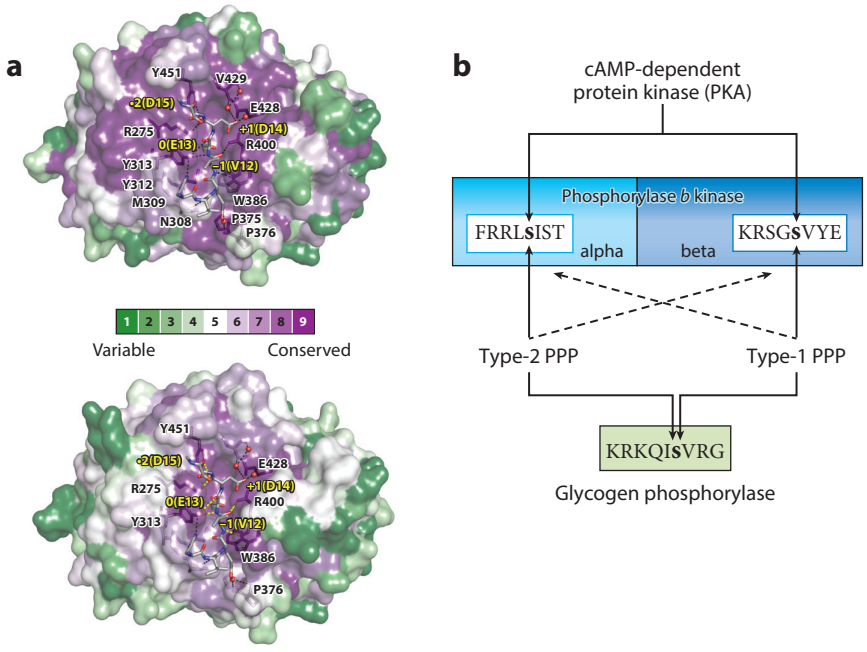

Figure 2

Substrate recognition by protein serine/threonine phosphatases (PPPs). (*a*) Three-dimensional structure of a chimeric protein phosphatase 5 (PP5) catalytic domain lacking the N-terminal tetratrichopeptide repeat domain but fused at its C terminus to the phosphomimetic S13E peptide from Cdc37, a selective PP5 substrate. The ^{9}HIEVEDD^{15} peptide occupies the hydrophobic and C-terminal substrate-binding grooves conserved in all PPPs to display the basis of substrate binding. The in-out-in-out peptide conformation has H^{9}, E^{11}, and E^{14} pointing inward, whereas I^{10}, V^{12}, and D^{15} point outward with the phosphomimetic residue E^{13} buried deep within the catalytic site. Catalytic site residues are conserved among 69 PP5 homologs (*top*) and in 87 different PPP domains (*bottom*). H bond interactions between substrate and enzyme conserved in PPPs are shown in yellow. Interactions of E^{11} and E^{14} occur via water-mediated H bonds within a spacious region of the hydrophobic groove, suggesting that a variety of side chains can be accommodated in positions −2 and +1 and water molecules adapt the substrates for different PPPs. The overall surface structure conserved between PP5 (*top*) and PPP (*bottom*) families is color coded as shown in the bar. (*b*) Biochemical criteria for classification of type-1 and type-2 PPPs were developed using the isolated catalytic subunits of PP1 and PP2A that showed distinct preferences for dephosphorylation of either the beta or alpha subunit of phosphorylase *b* kinase (PhK) at both sites phosphorylated by protein kinase A (PKA). Reactions are shown with solid arrows; dashed lines indicate relative lack of reaction. Subsequent studies of PP1 and PP2A holoenzymes confirmed this preference for beta and alpha subunits of PhK, respectively. By contrast, both PP1 and PP2A efficiently dephosphorylate glycogen phosphorylase, whereas other PPPs, such as PP3, show negligible activity against glycogen phosphorylase. Classification of newer PPP members, such as PP4, PP5, PP6, and PP7, is based on their sequences rather than these biochemical criteria.

phosphorylated Ser, and both subunits are efficiently phosphorylated in vitro and in vivo by PKA. As such, substrates of one kinase can be selectively dephosphorylated by different phosphatases. Sequence determinants for specificity of the kinase, such as N-terminal tandem basic residues and a hydrophobic residue [valine (Val) or isoleucine (Ile)] adjacent to the phosphoresidue, do not dictate specificity by phosphatases. The phosphorylation site in phosphorylase also has a pair of basic residues preceding Ser. Although not a substrate for PKA, it is a substrate for both type-1 and type-2A phosphatases. Other examples of intrinsic substrate specificity for catalytic subunits

of PP1 and PP2A come from studies of phosphoregulation of different proteins that have multiple sites of phosphorylation (96–98). More recently, PP6 substrate specificity was found to differ from both PP1 and PP2A, with apparent preference for pSer or pThr adjacent to acidic, not basic, residues, as seen in sites phosphorylated by casein kinases (54, 99, 100).

Even though some structural features in the catalytic sites of PPPs impart intrinsic specificity for substrates, there is compelling evidence that most PPPs do not exist as free catalytic subunits in cells but are associated with regulatory subunits and other proteins. Three-dimensional structures of PP1 complexes that catalyze dephosphorylation of different substrates, such as myosin light chains, glycogen-bound enzymes, ligand-gated ion channels, and eukaryotic initiation factors, clearly show that the PP1 catalytic subunit and, more specifically, the substrate-binding grooves do not undergo any discernable conformational change upon binding of regulator proteins. This indicates a dominant contribution by accessory or regulatory subunits, namely MYPT1, G_M, spinophilin, and GADD34, respectively, in the unique catalytic properties displayed by these heteromeric PP1 complexes.

2.2. PPP Regulatory Subunits and Short Linear Motifs

PPP catalytic subunits are distinguished from one another by their binding to co-conserved regulatory subunits and protein inhibitors specific for each PPP type. Like catalytic subunits, many of these PPP regulatory subunits have been highly conserved across evolution (e.g., Sds22, Tpd1, SAPSs). This indicates unique structural elements in these regulatory proteins may define their PPP specificity. Identification of nearly 200 PP1-binding proteins and availability of structures for numerous complexes containing the PP1 catalytic subunit and different PP1-binding domains have established sequences that mediate PP1 binding termed short linear motifs (SLiMs) (reviewed in 101, 102). Most SLiMs reside in unstructured regions devoid of secondary structure. The best-known SLiM for PP1 is the RVxF motif first identified in the skeletal muscle glycogen-binding subunit G_M, a subunit that binds PP1 and mediates recruitment to glycogen (103). Crystallography studies established that the side chains of the RVxF SLiM, particularly those of the hydrophobic residues Val and Phe, make contacts within a hydrophobic pocket on the PP1 surface (104). Thus, substituting for either Val or Phe in G_M or in other RVxF-containing proteins severely attenuates PP1 binding. Subsequent structural and mutagenesis studies highlighted other SLiMs, such as SILK, MyPhoNE, and SpiDoC, that are present in various PP1 regulators (105) and are conserved in a subset of PP1-binding proteins. These SLiMs associate with different regions on the PP1 surface such that the engagement of more than one motif provides for higher affinity and greater stability of PP1 complexes (**Figure 3**). Visualization of all known SLiM-binding sites on one PP1 catalytic subunit illustrates that almost the entire surface of the PP1 catalytic subunit is utilized for recruitment of regulatory proteins (78). Thus, some general rules have been crafted (106) for SLiMs that could be most useful for discovering new regulators of PP1 and possibly other PPPs. These rules include the following:

1. Each SLiM should be specific for a single PPP.
2. Each SLiM should be universal to enable identification of PPP regulators from different species.
3. SLiMs may be degenerate in their amino acid sequence to allow variations in affinities among different PPP regulators.
4. SLiMs should be flexible or nonexclusive such that combinatorial use of different SLiMs would allow for more than one regulator to be accommodated on any one PPP catalytic subunit.

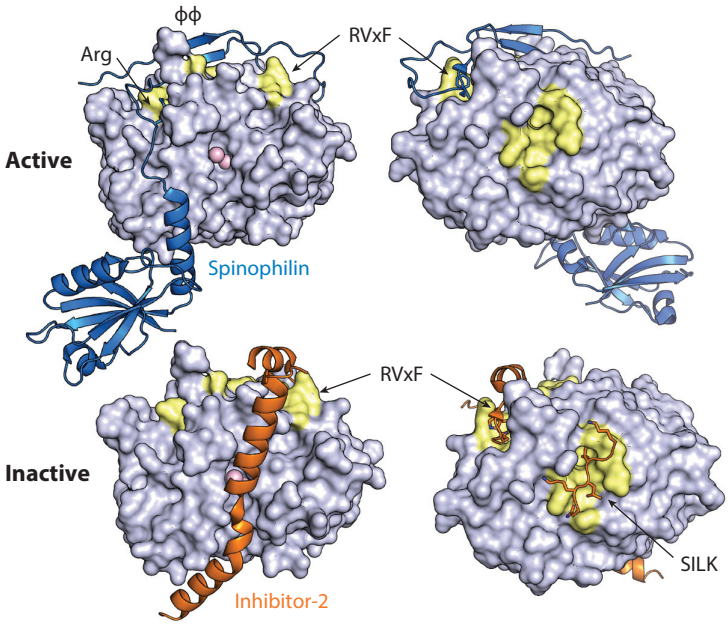

Figure 3

Association of protein phosphatase 1 (PP1) with a regulatory subunit and an inhibitor protein. Three-dimensional structures of PP1 alpha catalytic subunit bound to two distinct regulators, namely spinophilin (*blue*, *top panels*) and inhibitor-2 (*orange*, *bottom panels*). The front-facing catalytic site (*left*) contains two metals (*pink*), and the back surface (*right*) has one or more short linear motif (SLiM)-binding sites. These structures display how the PP1:spinophilin complex has an open active site, whereas the catalytic center is occluded in the PP1:inhibitor-2 complex. Spinophilin utilizes several SLiMs, including the arginine (Arg) motif, the ϕϕ sequence, and the RVxF motif to engage PP1, positioning its PDZ-binding domain in close proximity to the hydrophobic substrate-binding groove. Combining intrinsic substrate recognition of PP1 and the PDZ-binding domain allows the PP1:spinophilin complex to selectively recruit and dephosphorylate neuronal substrates such as AMPA receptors. By contrast, inhibitor-2 occupies the RVxF-binding site as well as an adjacent site occupied by the SILK motif and uses helical segments to traverse the hydrophobic groove to occupy the metal-containing catalytic site and inhibit PP1 activity. These proteins also can occupy different SLiM sites on PP1 to form a heterotrimeric inactive phosphatase complex containing PP1, spinophilin, and inhibitor-2.

Although these guidelines were developed primarily for PP1, they should be equally applicable for assembling the tool kit to identify regulators for other PPPs. Furthermore, although SLiMs appear to be widely conserved among the majority of the 200-plus PP1-binding proteins, some PP1 regulators, such as SDS22, do not appear to have any obvious SLiMs and associate with the PP1 catalytic subunit via structured domains such as helical leucine (Leu) repeats (107). Thus, a SLiM-based approach to identifying novel PPP complexes will not be exhaustive because it would exclude some PPP regulators.

Most attention has focused on SLiMs that define PP1 binding by regulators and substrates. However, type-2 PPPs are multimeric and, thus, employ a different strategy to recruit substrates. For example, PP2A forms a heterodimer (AC) containing a catalytic subunit (C) and a scaffold subunit (A), which in turn recruits a variety of regulatory B subunits to form ABC heterotrimers with distinct substrate specificities. Recent structural analysis of PP2A holoenzymes also demonstrates that B subunits can utilize SLiMs to recruit phosphoprotein substrates. Unlike PP1, the PP2A C subunit does not utilize a SLiM to bind the A subunit; instead it associates with specific

helical repeats in this scaffolding subunit (23). Mutations in PP2A A scaffolding subunits (108) or haploid insufficiency of PTPA (PPP1R4) are associated with human cancers. The PP3 phosphatase utilizes SLiM binding to both catalytic and regulatory subunits to recognize its substrates. PP4 forms dimers with PP4R1 and trimers with PP4R2/PP4R3 subunits (109). Currently, no three-dimensional structures of PP4 are available to identify the presence of SLiMs. Likewise, no three-dimensional structures have been solved of PP6 dimers containing SAPS, subunits that are conserved from yeast to human. In vertebrates, PP6 forms trimers with SAPSs and ANKRD subunits that contain ankyrin repeat domains, which are known in general to mediate protein-protein interactions, and there is evidence that ANKRD subunits contribute to substrate recruitment (100, 110). Solving three-dimensional structures of more PPP complexes will be a critical step toward identifying new SLiMs and their binding sites.

PP5 contains an N-terminal tetratrichopeptide repeat (TPR) domain and a C-terminal PPP catalytic domain. As TPR domains frequently bind other proteins, it was presumed that the N-terminal TPR domain mediates substrate recognition by PP5. However, biochemical studies show that PP5 is maintained in an inactive state via a C-terminal autoinhibitory sequence and activated following binding of HSP90 (111) to the TPR domain (23). HSP90 in turn recruits Cdc37, a cochaperone, which is a PP5 substrate (112). Thus, HSP90 either directly or in association with Cdc37 recruits client proteins, including many protein kinases that are PP5 substrates. Complementary to the intrinsic substrate specificity demonstrated by the PP5 catalytic domain, HSP90 functions, in effect, as a PP5 regulatory subunit that is equally critical for the recognition of substrates.

2.3. Engaging PP1 with Different Short Linear Motifs

Short linear sequences that mediate binding to PP1 were first found from studies of the intrinsically disordered protein phosphoinhibitor-1. In determining the primary structure of rabbit skeletal muscle inhibitor-1 (I-1), Aitken and colleagues (113, 114) assayed the PP1 inhibitory activity of several I-1 phosphopeptides, revealing a KIQF sequence that distinguished the active from inactive I-1 phosphopeptides. Mutations of I-1 (115) and the structurally related PP1 inhibitor DARPP-32 (116) established that the KIQF motif and, more specifically, the two hydrophobic residues Ile and Phe were critical for PP1 binding. These data led to the development of a two-site model for PP1 binding and inhibition, requiring both KIQF and the pThr site. The distance between the $_{9}$KIQF$_{13}$ sequence and pThr$_{35}$ suggests that the SLiM binding site is some distance from the PP1 catalytic center that binds pThr$_{35}$, where phosphoinhibitor-1 (and pDARPP-32), by virtue of poor reactivity as a substrate, inhibits PP1.

Comparison of the primary sequences of the glycogen-targeting subunits G_L and G_M, isolated from liver and skeletal muscle, respectively, identified a conserved 25-residue sequence (117), and assays with synthetic peptides representing this sequence established it as a PP1-binding site. Subsequent X-ray crystallography of PP1c with a bound G_M peptide (93) revealed the RVSF sequence docked in a surface hydrophobic pocket on PP1 that was some distance from its bimetallic catalytic center. The RVxF-binding sequence was subsequently identified in the smooth-muscle myosin-binding subunit MYPT1 (118), I-2 (67), spinophilin (119), NIPP1 (120), PNUTS (121), GADD34 (122), Repo-Man, and KI-67 (123). Three-dimensional structures of these PP1 complexes showed that the RVxF SLiM in these PP1 regulators resides within inherently unstructured domains (124). The solution structure for I-2 showed that it was almost entirely an intrinsically disordered protein (125), which acquired secondary structure following PP1 binding (67). The structures of multiple PP1 complexes illustrated the diversity of SLiM sequences that were accommodated in the RVxF-binding hydrophobic pocket. This diversity likely translates into a range of

binding affinities that these regulatory subunits display for PP1 and suggests a hierarchy for PP1 binding among the 200-plus known PP1-binding proteins (105).

The structures of PP1 regulators, such as spinophilin, and inhibitors, such as I-2, show that both utilize an RVxF SLiM (**Figure 3**); yet, by utilizing other SLiMs, they also display unique modes of PP1 engagement. Thus, in the PP1:spinophilin complex (119), the PP1 catalytic center is available for substrate binding, consistent with the formation of active phosphatase complex. By contrast, in the PP1:I-2 complex, the catalytic site is occluded (67), consistent with an inactive phosphatase complex. These PP1 complexes result from the engagement of PP1 with one dominant SLiM, such as the RVxF motif. An additional SLiM then stabilizes the association with PP1 while also defining the overall architecture of the PP1 holoenzyme. The physicochemical principle involved is that the overall dissociation constant for separation of these proteins (in the low nanomolar range) is the product of the dissociation constants for the individual interactions ($K_{d\ overall} = K_{d1} \times K_{d2}$). Thus, once formed, the PP1:regulatory subunit complex or holoenzyme is relatively stable and able to interact with substrates. There also are a few examples of direct PP1 binding to phosphoprotein substrates that possess their own RVxF docking sequences, such as retinoblastoma protein (126), yeast translational initiation factor Sui2 (127), and select PP2A B subunits (128). Nevertheless, the primary experimental strategy for identifying potential PP1 substrates is to define the regulatory subunit of a specific cellular PP1 complex. Once that information is obtained, phosphoproteins recruited or scaffolded by a given regulatory subunit may be investigated.

Most recent attempts to isolate PPP complexes have relied on either co-immunoprecipitation with PPP catalytic subunits or affinity isolation of PPP holoenzymes on immobilized toxins such as microcystin-Sepharose (129) or microcystin-biotin conjugate (130). Mass spectrometry is then used to identify PPPs and their associated regulatory proteins. Digoxygenin-PPP conjugates have been used as probes in far-Western blots (overlays) to identify putative PPP regulators (131). However, this latter approach has realized only moderate success and generally only with the most highly abundant PPP regulators. Use of RVxF peptides to displace potential PP1-binding proteins has also met with only limited success (132, 133), perhaps because any individual SLiM in the form of a short synthetic peptide shows relative low affinity (micromolar range) for the target PPP. Thus, SLiM peptides are unlikely to effectively compete with regulatory proteins that bind PP1 via multiple SLiMs. Bioinformatic approaches using any single SLiM may also not provide a sufficiently stringent filter for identifying candidate PPP regulators. For example, the RVxF sequence is present in nearly 10% of proteins encoded by the mammalian genome (105). To counter this, efforts have been made to assess the conservation of sequences surrounding shorter SLiMs and to define the tolerance or degeneracy of sequences that can be accommodated in binding the target PPP. For example, comparison of sequences in validated PP1 regulators has expanded the RVxF motif to provide a more refined definition, namely $[K_{55}R_{34}][K_{28}R_{26}][V_{94}I_6]\{FIMYDP\}[F_{83}W_{17}]$, where subscripts indicate the percentage of total examples in which an individual residue is found in any given position. This reveals the dominance of Val and Phe in this PP1-binding sequence. Including segments found in other highly conserved SLiMs, such as ϕϕ (120) and the Arg motif (121), that lie C terminal to the RVxF motif can further extend the motif. Use of such extended sequences for conserved SLiMs increases the stringency of searches and should improve the identification of PP1 regulators.

Because the RVxF motif is prevalent in PP1 regulators and has a defined binding site on the PP1 catalytic subunit, it would be logical to think that the RVxF motif would allow only one PP1 regulator to bind the PP1 catalytic subunit at any one time. However, PP1 can recruit more than one regulator, each utilizing different SLiMs. Indeed, biochemical and structural studies have confirmed the existence of several trimeric PP1 complexes including, for example, PP1, spinophilin, and I-2 (134, 135). X-ray crystallography of this complex shows that I-2 relinquishes its

association with the RVxF-binding pocket, which is instead occupied by the RVxF of spinophilin. Other SLiMs in I-2 such as SILK allow I-2 recruitment to PP1. In addition, direct interactions between the two PP1-binding partners spinophilin and I-2 enables formation of the trimeric complex. There is experimental evidence for other PP1 trimers: PP1, G_M, and I-2 (136); PP1, GADD34, and I-1 (137); PP1, LMTK2, and I-2 (138); PP1c, Nek2, and I-2 (139); PP1, SDS22, and I-3 in mammals (83); and YPI1, SDS22, and Glc7 in yeast (140). The three-dimensional structures of these complexes remain unknown, but their solution undoubtedly will reaffirm that different SLiMs are used for simultaneous docking of two partners to PP1.

Because regulatory or targeting subunits narrow the substrate specificity of PP1 complexes, they have been labeled inhibitory subunits in the human genome (25). However, as discussed in Section 2.3 (also see **Figure 3**), regulatory subunits like spinophilin do not completely block substrate access to the PP1 catalytic site and therefore are not bona fide inhibitors. However, juxtaposition of the PDZ domain of spinophilin to the substrate binding channel of the PP1 catalytic subunit provides preferential access to proteins containing the requisite PDZ-binding motif. Thus, the PP1:spinophilin complex selectively dephosphorylates AMPA receptors (141, 142). In a comparable manner, MYPT1 also positions its ankyrin repeats adjacent to the PP1 catalytic face to expand and modify the substrate binding site (118). Thus, the PDZ domain enables dephosphorylation of AMPA and NMDA receptors by PP1 bound to spinophilin and neurabin (142), and PP1 dephosphorylates the light chain of myosin complex that is recruited to the ankyrin repeats of MYPT1. Recent studies show that the ability of PP1 to dephosphorylate the eukaryotic translation initiation factor eIF2α is dependent on the scaffolding of both eIF2α at adjacent but independent sites on GADD34 (122).

Some PPP regulatory subunits possess structural elements that define their subcellular localization or association with specific organelles. By localizing PPPs, these regulatory subunits increase the local concentration of PPP in specific subcellular compartments to facilitate the dephosphorylation of phosphoproteins present in that location. Neurabins target PP1 to actin-rich postsynaptic density in dendritic spines (143) to regulate spine morphology and maturation (144). Several different glycogen-targeting subunits localize PP1 to glycogen granules (145) to facilitate hormonal control of glycogen storage. Localization of PP1 to the endoplasmic reticulum via its association with GADD34 (146) positions the phosphatase to control the unfolded protein response (147).

Even with more than 200 regulatory subunits, there is a high likelihood that each PP1 complex dephosphorylates multiple phosphoprotein substrates. Plus, in any one phosphoprotein, PP1 probably dephosphorylates only some phosphorylated sites. Accordingly, more than one PPP will be required for complete dephosphorylation of multiply phosphorylated proteins. During mitosis, MYPT1 recruits PP1 to centromeres (148, 149) where PP2A is also recruited via its association with Shugoshin (150) and BubR1 (151). These different PPPs dephosphorylate various phosphoproteins, namely PLK1, Ssc1, and Aurora B, to facilitate sister chromatid separation. This raises an important question or challenge for future studies. How do different PPP complexes communicate with each other to ensure systematic or orderly dephosphorylation of sites or substrates to control cell division?

2.4. PP1 Isoform Selectivity and Specificity

The ability of regulatory subunits to discriminate among the four mammalian PP1 isoforms is still poorly understood. In mammals, three genes, α, β, and γ, encode PP1. PP1γ mRNA is spliced to produce isoforms PP1γ1 and PP1γ2, the latter of which is expressed predominantly in testes. These isoforms differ primarily in their N- and C-terminal sequences that are not seen in PP1 crystal structures. Thus, how spinophilin and neurabin preferentially associate with

PP1γ1 is unknown (152). MYPT1 binds exclusively to PP1β (153), whereas GADD34 and tensin prefer PP1α (146, 154). It is notable that PP1γ-null mice are viable but male sterile, consistent with a major role for PP1γ2 in testes (155). Analysis of PP1 complexes in mutant mouse tissues showed that, in the absence of PP1γ1, spinophilin and neurabin form complexes with PP1α (152). Biochemical studies demonstrated that some PP1 regulatory subunits are unable to distinguish among different PP1 isoforms in vitro, showing nearly identical affinities for recombinant PP1α, β, and γ1 isoforms (121). Yet, other studies suggested that sequences flanking the RVxF motif in spinophilin and neurabin impart some degree of isoform selectivity (156). Recent structural analyses of PP1γ1 complexes containing the mitotic PP1 regulators Ki-67 and Repo-Man (123) highlighted the critical role of a single amino acid, Arg_{20}, near the N terminus of PP1γ1 in isoform selectivity of these regulators. More specifically, substitution of Arg_{20} in PP1γ1 with a residue found in the same position in another PP1 isoform reduces its affinity for Ki-67 and Repo-Man, whereas introduction of Arg_{20} into the isoform PP1α enhances its association with the same regulators. Though Arg_{20} does not directly bind the regulatory subunits, data show that Arg_{20} participates in the generation of an ordered pocket that is uniquely observed in PP1γ1 and binds Ki-67 and Repo-Man. Thus, structural determinants in both regulatory and catalytic subunits may contribute to the selectivity of regulatory subunits for specific PP1 isoforms. Notably, Kumar et al. (123) defined a novel PP1-binding SLiM, termed Kir-SLiM, present only in Ki-67 and Repo-Man, but how this selectively recognizes PP1γ remains unknown.

Functional studies, such as replacing the single yeast PP1 (Glc7) with distinct human PP1 isoforms, showed that all yeast strains expressing human PP1 were viable, but there were significant differences in the regulation of specific Glc7-regulated processes in yeast (60). This characteristic was partly explained by the inability of some human PP1 isoforms to recruit endogenous yeast PP1 regulatory subunits. Thus, despite their 80% sequence identity with yeast PP1, all human PP1 isoforms perform less effectively than does yeast Glc7 in controlling various yeast functions. This further supports the idea that both the PP1 isoform and its specific regulatory subunits determine PP1 cellular functions, as defined by dephosphorylation of specific substrates. In this regard, *S. cerevisiae* remains the only organism with a single PP1 gene, whereas other fungi, such as *S. pombe* and *Aspergillus nidulans*, have two PP1 genes. *Arabidopsis thaliana* has nine PP1 genes (157). *C. elegans* expresses 15 PP1 isoforms, 12 of which are most abundant in testes (158). By contrast, 6 of 10 *D. melanogaster* PP1 isoforms are expressed solely in testes (159), whereas the two PP1 genes located on the Y chromosome are not expressed in female flies (160). The presence of multiple PPP genes allows for tissue-specific expression of different PP1 isoforms. The physiological relevance of PPP isoform distribution remains to be determined. Together, these studies show that PP1 isoforms are not fully equivalent, and subtle (unknown) differences in structure give rise to the functional uniqueness of individual PP1 holoenzymes.

2.5. Short Linear Motifs Dock to PP2A Regulatory Subunit

The predominant form of PP2A is a heterotrimeric complex comprised of one catalytic subunit (C), one scaffolding subunit (A or PR65), and one regulatory B subunit (B, B′, B″, or B‴) (161–163). The PP2A heterotrimer as the preferred or obligatory arrangement is a concept based on RNAi studies in *Drosophila* cells where knockdown of C, A, or multiple B subunits depleted the other subunits, suggesting an interdependency of the three subunits (164). However, earlier analyses by Walter and colleagues (165) provided convincing evidence that approximately one-third of PP2A in mammalian cells exists as an AC dimer. Protein census of human cell lines by mass spectrometry estimates that the number of copies per cell is unequal with A > C > B subunits, which is consistent with cells possessing both dimers and trimers because AC outnumbers B. This

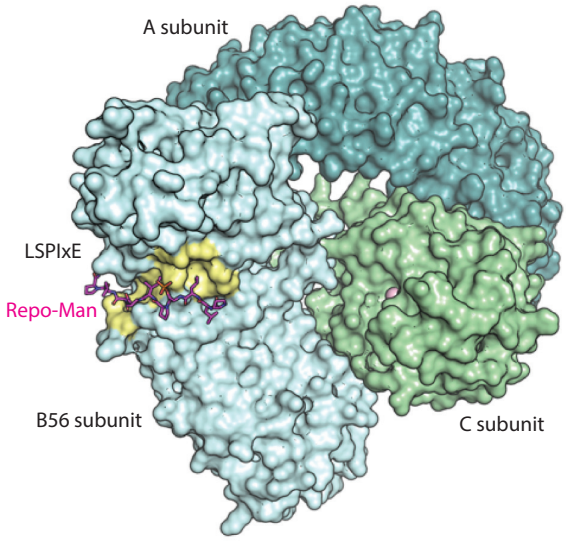

Figure 4

Short linear motif (SLiM) docking to protein phosphatase 2A (PP2A) regulatory B subunit. The structure of the heterotrimeric PP2A holoenzyme comprising a catalytic (C) subunit, scaffolding A subunit, and B56 regulatory subunit is shown bound to a peptide SLiM from Repo-Man, which is also a PP1-binding protein. This assembly targets phosphatases to chromosomes, and this structure defined the LSPIxE SLiM for PP2A that also appears in BubR1, a protein that targets the PP2A holoenzyme to centromeres. Interestingly, phosphorylation of Repo-Man or BubR1 near this SLiM enhances their binding to PP2A, although these sites are probably not directly dephosphorylated by PP2A. Instead, recruitment of PP2A to these proteins positions the phosphatase in proximity to other mitotic substrates that remain unidentified. The LSPIxE SLiM has been used to identify more than 100 potential PP2A binding proteins, some of which may be substrates of B56-containing holoenzymes. SLiMs may similarly facilitate identification of regulators or substrates for PP2A holoenzymes containing other B subunits.

is important because B subunits play a dominant role in defining the substrate specificity of PP2A (166). The precise function of the AC dimer in cell signaling remains unclear, but we speculate it serves as a readily available pool for assembly of different heterotrimers in response to signaling.

Association between the PP2A heterotrimer containing the B56 subunit and the kinetochore protein BubR1 utilizes a conserved sequence that Plk1 and Cdk1 phosphorylate to enhance PP2A binding (151, 167). Structural analyses established that the LXXIXE sequence binds to the concave surface of B56 pseudo-HEAT repeats 3 and 4. This PP2A site differs from that bound by another PP2A regulator, Shugoshin, that associates with kinetochores (168). Crystallography of the B56-containing PP2A complex with the nuclear scaffolding protein showed that Repo-Man (123) and BubR1 have a similar B56-binding sequence (**Figure 4**). Phosphorylation of this region in both phosphoproteins results in enhanced binding to HEAT repeats 3 and 4 in PP2A. These studies define LSPIxE as a SLiM for heterotrimeric PP2A (AB56C). Using the more expanded and degenerate motif [LCVMIF]-SPIxE, Wang et al. (169) identified 70 potential B56-binding partners in the human proteome. Wu et al. (170) used a phage display library to establish the diversity of peptide sequences that bind B56, and with this expanded list of SLiMs, they identified more than 100 potential B56-binding proteins, including Kif3A and KNL1, as well as Cyk4, whose phosphorylation by Polo-like kinase is reversed by the B56-containing PP2A phosphatase during cytokinesis. The B56-binding SLiM was also found in Cip2A, a PP2A inhibitor that is overexpressed in human cancers (171). These data hint at a novel mechanism for PP2A inhibition

by which Cip2A utilizes its SLiM to displace B56-binding proteins and abrogate PP2A activity in cancer cells. These and other studies further suggest that many B56-associated proteins, including PP2A substrates such as Cyk4 (170) and KNL1 (172), share the B56 SLiM. New bioinformatics tools, such as SLiMSearch (173) and Pro-PD phage display libraries (163), were instrumental in identifying the PP2A B56 SLiM. When utilized in conjunction with the continuously expanding information on the human phosphoproteome (27), these experimental approaches should accelerate the discovery of PP2A substrates and regulators (170, 174, 175).

X-ray crystallography of the PP2A B55-containing holoenzyme (176) and structure-function analysis of Tau dephosphorylation highlighted two sequences in the Tau protein that were predicted to bind the B55 subunit. Further studies will be required to establish the precise Tau sequences that interact with B55. Researchers will then be able to identify SLiMs that can provide new insights into substrate recognition by different PP2A holoenzymes.

2.6. Dual Short Linear Motifs for PP3

PP3 (also known as Calcineurin) is a dimer comprised of an A subunit that contains a PPP catalytic domain and a Ca^{2+}-binding regulatory B subunit. An autoinhibitory segment in the A subunit obstructs the active site to maintain this phosphatase in an inhibited state. Following Ca^{2+}-dependent binding of calmodulin, PP3 undergoes a conformation change that displaces the autoinhibitory domain and results in activation of this phosphatase (177). The blockbuster immunosuppressive drugs cyclosporin and FK506 are natural products that have been used for many decades in patients who have undergone organ transplantation. These drugs associate with immunophilins that are *cis-trans* peptidyl prolyl isomerases, generating a drug-protein complex that inhibits PP3. Inhibiting PP3-catalyzed dephosphorylation of the transcription factor NFAT and members of the NFAT family that represent the major PP3 substrates in mammalian tissues mediates the immunosuppressive activity of these drugs. Following identification of the PP3-binding site in NFAT, short peptides were used to displace NFAT from PP3 and thus established the PIXIT sequence as the primary SLiM found in PP3 substrates (178). The PIXIT docking site was mapped to the PP3 catalytic A subunit (179), and co-crystallization of a PIXIT peptide with PP3 (180) highlighted the conservation of the PP3 PIXIT-binding site (reviewed in 23).

In *S. cerevisiae* lacking a functional PP3, analysis of hyperphosphorylated proteins identified numerous potential PP3 substrates with a PIXIT SLiM (181). However, the PIXIT motif is present in PP3 substrates as well as in scaffolding proteins (e.g., AKAP79) and PP3 inhibitors (e.g., RCAN1). Small molecules that displace the PIXIT peptide from NFAT were screened to identify PP3 inhibitors that effectively block PP3-NFAT signaling in T cells (182). These SLiM-blocking compounds may lead to the development of novel immunosuppressive drugs.

In addition to the PIXIT sequence, NFAT contains an LxVP motif that appears in many mammalian PP3 substrates but only in one yeast protein, RCN1 (177). Synthetic peptides that occupy the LxVP site located near the active site inhibit PP3-mediated dephosphorylation of substrates in vitro. Structural studies of PP3 bound to a viral protein inhibitor established that this protein occupies both PIXIT and LxVP sites (**Figure 5**) and blocks substrate binding (183). Structural studies of PP3 bound to FK506 (71, 76) and cyclosporin (184) established that drug-immunophilin complexes bound to the composite site formed by the A and B subunits that was otherwise occupied by the LxVP sequence. This demonstrates that the LxVP SLiM is critical for recruiting substrates and drug-mediated occupancy of this SLiM site potently inhibits PP3-mediated dephosphorylation of NFAT and other PP3 substrates. Together, these data illustrate another benefit of identifying PPP SLiMs: understanding the binding sites of PPP substrates and regulators will likely enable the future development of novel and selective PPP inhibitors. Finally,

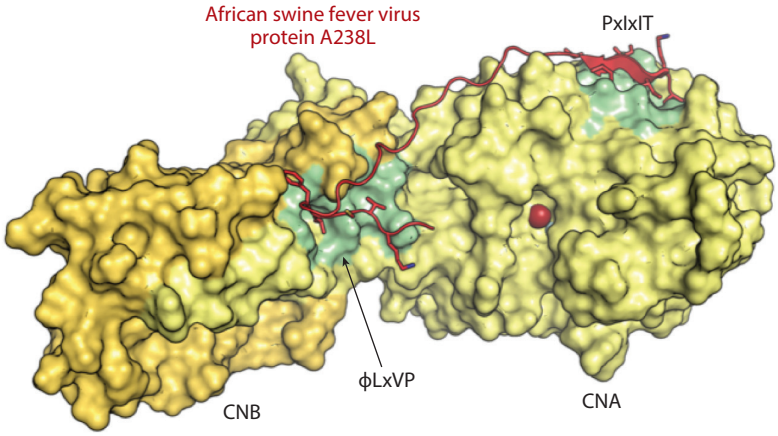

Figure 5

Protein phosphatase 3 (PP3) uses short linear motifs (SLiMs) for substrate recognition. Shown are the AB structure of the PP3 holoenzyme, also known as calcineurin or PP2B, in complex with the inhibitor protein A238L from African swine fever virus; a holoenzyme containing the catalytic A subunit (CNA) with catalytic metals (*red*); and the regulatory B subunit (CNB) bound to a portion of the viral protein containing two SLiMs, PXIxIT and ϕLxVP. The SLiM binding site for ϕLxVP is created at the interface of the A and B subunits and is occupied either by mammalian PP3 substrates or by the protein-bound immunosuppressive drugs cyclosporin and FK506 that inhibit PP3 activity. The ability of the A238L protein (*orange*) to occupy both substrate recognition sites suggests that it inhibits PP3 by preventing substrate binding. The PxIxIT and ϕLxVP motifs have been used to identify potential PP3 substrates in both yeast and mammalian cells.

Sheftic et al. (185) identified more than 500 potential PP3 substrates and significantly expanded our understanding of the PP3 signaling network in human cells. Their study used an extended SLiM sequence, πϕLx[VPLHI]x, and additional filters, including a PIXIT motif, propensity for intrinsic disorder, and pSer or pThr.

2.7. Short Linear Motifs for Other PPPs?

Whether the experimental approaches that successfully identified SLiMs for PP1, PP2A, and PP3 will work for other PPPs, i.e., PP4, PP5, PP6, and PP7, is currently unclear. Perhaps the simplest of these PPPs are PP5 and PP7, widely considered as monomeric enzymes, but both are inactive in their basal state owing to the presence of autoinhibitory domains. Thus, either other proteins or factors need to be present to activate these proteins, or the autoinhibitory domains must be deleted to generate active forms of these enzymes that could be used to identify potential substrates.

Several regulatory subunits for PP4 and PP6 are known, but how they recognize substrates has not been established. At least one case indicates there may be multiple sites for interactions between PP6 SAPS and its substrate (186). As with PP2A, cocrystallization of catalytic and regulatory subunits may be a necessary step to identify or confirm SLiMs that mediate substrate recognition for PP4 and PP6.

3. POSTTRANSLATIONAL MODIFICATIONS AND PPPs

3.1. Regulation of PPPs by Phosphorylation

Phosphorylation plays a fundamental role in the regulation of most PPPs. Here we highlight three modes for suppression of PP1 and PP2A activity that include phosphorylation of PPP

catalytic subunits, regulatory subunits, and inhibitor proteins. All three suppress phosphatase activity and thus provide reciprocal links between changes in phosphatase activity and kinase activity. In general, phosphatases are inhibited when kinases are activated. This results in enhanced kinase signaling with increased signal amplitude and duration.

The first mode of PPP regulation involves direct phosphorylation of PPP catalytic subunits in their C terminal tails that results in their transient inhibition. Thus, PP1 is phosphorylated by multiple Cdks at Thr320 in a conserved TPPR sequence near the C terminus of the PP1 catalytic subunit, present in different isoforms (187, 188). This C-terminal sequence might occupy the C-terminal groove in the PP1 catalytic subunit and insert pThr into the active site to produce PP1 inhibition. This notion is consistent with the observation that the pThr320 undergoes slow autodephosphorylation that reactivates PP1. Similarly, PP2A is phosphorylated by different kinases at Thr305 (189) or Tyr307 (190) in the C-terminal TPDYFL sequence. As a result, enzyme activity is reduced, and self-dephosphorylation reverses this reduction. Dephosphorylation of pTyr307 in PP2A is increased in Alzheimer's disease (191) and enhanced by the PP2A regulator PTPA through activation of the reaction with pTyr phosphatase, PTP-1B (192). Thus, multiple mechanisms are involved in the dephosphorylation and reactivation of the phosphorylated PP2A catalytic subunit.

The second mode of PPP regulation is mediated by phosphorylation of PPP regulatory subunits that can either suppress or enhance PPP activity. Phosphorylation of myosin phosphatase subunit MYPT1 at Ser695 inhibits bound PP1 (193). Reduced activity of the myosin phosphatase likely involves binding of pSer695 at the PP1 active site where its poor reactivity as a substrate results in PP1 inhibition. Another example involves phosphorylation of Ser67, which lies within the SLiM RVSF sequence in the skeletal-muscle glycogen-targeting subunit G_M (194). Phosphorylation interferes with G_M binding to PP1, thereby inhibiting assembly of glycogen-bound phosphatase (195). This illustrates the potential for control by phosphorylation of Ser (and possibly Thr) present within PNUTS (196) or near SLiMs in other PP1 regulators. In contrast, phosphorylation of SDS22 by PLK1 strengthens PP1 binding but inhibits PP1-mediated dephosphorylation of Thr232 on Aurora B (197). Thus, phosphorylation can modulate the assembly or disassembly of PP1 complexes. Mass spectrometry has identified more than 50 phosphorylated residues in the PP1 regulatory subunit GADD34 (198), even though full coverage of the GADD34 primary sequence was not attained (the central PEST repeats are highly phosphorylated but were not captured by mass spectrometry). There could be as many as 100 PTMs of GADD34, an extraordinary number that presents an enormous challenge in deciphering how phosphorylation contributes to the control of PP1 functions.

Phosphorylation of B subunits both positively and negatively modulates PP2A activity. For example, phosphorylation of the B56α regulatory subunit at Ser41 by protein kinase C (PKC) reduces PP2A activity (199). Conversely, phosphorylation of B56δ by PKA activates PP2A (200), and PKR-mediated phosphorylation of B56α enhances the phosphatase activity of the PP2A holoenzyme containing this B subunit (201). Conformational changes transmitted between subunits of the heterotrimer seem to affect catalytic activity. MAPK phosphorylates the B56γ1 subunit at Ser327, which is highly conserved among other B56 family members, results in the dissociation of the B subunit from the PP2A holoenzyme, and thereby reduces the amount of this phosphatase that counteracts MAPK signaling (202). Similarly, Bα subunit may be phosphorylated at Ser167 during mitosis to disrupt its association with the PP2A AC dimer (189). Although by no means an exhaustive list, these phosphorylations of PP2A B subunits highlight how covalent modifications can regulate PP2A activity. The phosphoproteome (27) (**https://www.phosphosite.org**) provides many examples of Ser, Thr, and Tyr phosphorylation on PPPs and their regulatory proteins. The functional consequences of most have not yet been analyzed. We envision a broad network of

phosphorylation-dephosphorylation reactions that regulate PPP functions. These reactions will need to be incorporated into the wiring diagrams for signaling networks.

The third mode of PPP negative regulation is represented by heat-stable protein inhibitors, which might be considered as auxillary subunits for PP1, PP2A, and calcineurin. Multiple inhibitors, most of which are phosphorylation dependent, selectively inhibit either PP1 (I-1, CPI-17, DARPP-32, PHI-1) or PP2A (ENSA, SET, ARPP-16, CIP2A). Furthermore, protein inhibitors can be selective for individual holoenzymes of each PPP type, for example, CPI-17 for MLCP and ENSA for AB55C. These phosphoproteins are in essence poor substrates with slow kinetics of dephosphorylation that occupy the PPP catalytic site in lieu of true substrates, in what was recently called "inhibition by unfair competition" (203, 204). The RCAN family of PP3 inhibitors are phosphorylated at multiple sites and reduce PP3 phosphatase activity (205). The phospho-dependent PPP inhibitors have important physiological roles in responding to exogenous or endogenous signaling: They promote pharmacological calcium sensitization of smooth muscle (CPI-17 and MYPT1/PP1) as well as entry into mitosis during the cell division cycle (Ensa/ARPP-19 and PP2A). Increased expression of the PP2A inhibitors SET and CIP2A (reviewed in 206) is observed in different types of human cancers. Blocking the actions of PPP inhibitors with pharmaceutical agents is a novel approach to cancer therapy now under development in academia and industry.

3.2. Methyl-Esterification of PPPs

Different type-2 PPP phosphatases undergo methyl-esterification (commonly and incorrectly called methylation), as first found on the C-terminal Leu of the PP2A catalytic subunit (reviewed in 207). LCMT-1 (leucine carboxymethyl transferase-1) (208) catalyzes the reaction with *S*-adenylmethionine as donor substrate. This modification is reversible, and the highly specific esterase PME-1 (protein phosphatase methylesterase-1) (209) catalyzes demethylation. The prevailing evidence suggests that methyl-esterification of the PP2A catalytic subunit affects the assembly of PP2A heterotrimers that contain certain B subunits in both yeast (210) and mammalian cells (211–215). Studies showed enhanced activity of LCMT-1 with the PP2A AC dimer versus LCMT-1 with the isolated PP2A catalytic subunit, positing a role for the scaffolding A subunit in promoting PP2A methyl-esterification (216). LCMT-1 is the major enzyme that catalyzes methyl-esterification of not only PP2A but also PP4 and PP6 catalytic subunits (217). On the basis of results with PP2A, we might expect LCMT-1 to promote the assembly of multisubunit complexes for multiple PPP types. Loss of function of LCMT-1 results in reduction in cellular content of PP4-PP4R1 and selected PP2A complexes, emphasizing the role of holoenzyme assembly in stabilizing PPP catalytic and regulatory subunits. Some studies have indicated that Tyr-307 phosphorylation of the PP2A catalytic subunit negatively regulates PP2A methyl-esterification (218), suggesting phosphorylation not only inhibits PP2A activity but also reduces cellular PP2A holoenzyme levels. The haploinsufficiency of PTPA (also known as PPP2R4) (219) or elevated PME-1 levels (220) in human cancers reduces PP2A methyl-esterification and activity. Similarly, increased expression of the PP2A inhibitor SET seen in cancer cells was associated with reduced methyl-esterification of PP2A and reduction in overall PP2A activity (221), demonstrating that more than one PTM can be utilized in downregulation of PP2A function contributing to human disease.

3.3. Cross Talk Between PPPs and Protein Acetylation

Mass spectrometry has found extensive PTMs, including phosphorylation, acetylation, methyl-esterification, and ubiquitination, in mammalian proteins. Our discussion here is focused on

emerging evidence for acetylation and ubiquitination of PPPs and potential mechanisms for cross talk between phosphorylation, acetylation, and ubiquitination in cell signaling (222).

Histone deacetylases (HDACs) and PP1 phosphatase act in concert to govern acetylation of lysines (Lys) in histones and tubulin. Indeed, the increase in histone acetylation seen after HDAC inhibition was remarkably similar to that following PPP inhibition, suggesting some coordination of PPP and HDAC activities (223). Studies in yeast showed that mutations in Glc7 (PP1) increase histone H3 phosphorylation (Ser10) while H3 acetylation (Lys9) decreases Ser10 phosphorylation, illustrating cross talk between Ser phosphorylation and histone acetylation (224). Canettieri et al. (225) studied PKA phosphorylation of transcription factor CREB (Ser133) and observed recruitment of histone acetyltransferases by phospho-CREB and acetylation of promoter-bound histones to accelerate CREB-mediated gene transcription. Unexpectedly, these studies found HDAC inhibitors enhanced CREB phosphorylation. Recruitment of HDAC1 to the promoter enhanced PP1-mediated CREB dephosphorylation. Subsequent biochemical studies (226) established that PP1 physically associates with multiple HDACs and demonstrated that HDAC inhibitors disrupt these HDAC/PP1 complexes. Histone signaling mediated by HDAC1/PP1 complexes regulates gene transcription by CREB as well as by BRD4 and P-TEFb (227), and the ability of PP1 to regulate the histone code may be critical for long-term memory in the mammalian brain (228).

HDACs working in concert with PPP phosphatases regulate tubulin acetylation. Levels of the PP1 inhibitor I-2 also impact tubulin acetylation (229), probably by targeting the HDAC6/PP1 complex. The MYPT1/PP1 myosin phosphatase communicates with HDAC6 to modulate microtubule acetylation and contractility associated with cell migration (230). Myosin phosphatase also binds and dephosphorylates HDAC7 (231). Acetylation of microtubules is highly responsive to changes in ectopic expression of different PP2A B subunits (232). Data indicate B55α binds to HDAC5 and promotes its PP2A-mediated dephosphorylation (233). PP2A also dephosphorylates HDAC4 (234) and HDAC7 (235). Acetylation can play a direct role in controlling PPP function, as evidenced by acetylation of the PP2A-interacting protein BubR1 to enhance binding to PP2A-B56. This is essential for PP2A-mediated suppression of Aurora B, which is subject to acetylation (236). HDAC3, likely through its association with a PP2A holoenzyme, enhances PP2A-mediated dephosphorylation of STAT3 (pSer727) (237). Remarkably, binding of PP4/PP4R1 to HDAC3 suppresses its deacetylase function (238). All these findings demonstrate the extensive interplay between PPP phosphatases and deacetylases.

Conversely, protein acetylation modulates substrate dephosphorylation by PPPs. Insulin and glucagon regulate acetylation of phosphorylase at Lys470, and this acetylation enhances interaction with the PP1 regulatory subunit G_L to facilitate dephosphorylation by G_L-bound PP1 (239). Translational initiation factor eIF2α is phosphorylated (Ser51) and acetylated (Lys141/143) following endoplasmic reticulum stress in mammalian heart (240). These studies showed that the NAD-dependent protein deacetylase SIRT1 binds eIF2α and functions as an eIF2α deacetylase. More recent work demonstrates that SIRT1 also binds the PP1 regulator GADD34 (241). In turn, GADD34 binds both PP1α and eIF2α to assemble a stress-activated eIF2α phosphatase that recruits the inactive, phosphorylated form of SIRT1 in response to oxidative stress and dephosphorylates SIRT1 (at Ser47) to activate it as a deacetylase. SIRT1 deacetylation of eIF2α then facilitates its dephosphorylation and reactivation by GADD34/PP1 (**Figure 6**). Loss of function of either SIRT1 or GADD34 prolongs eIF2α phosphorylation and contributes to stress-induced cell death. Mass spectrometry demonstrates that GADD34 and PP1C are acetylated, but the effects on function are currently unknown.

Comparison using stable isotope labeling and quantitative mass spectrometry of acetylated proteins from wild-type and SIRT1-null murine embryonic fibroblasts has identified more than 4,500 acetylated Lys residues in approximately 1,800 acetylated proteins that are potential SIRT1

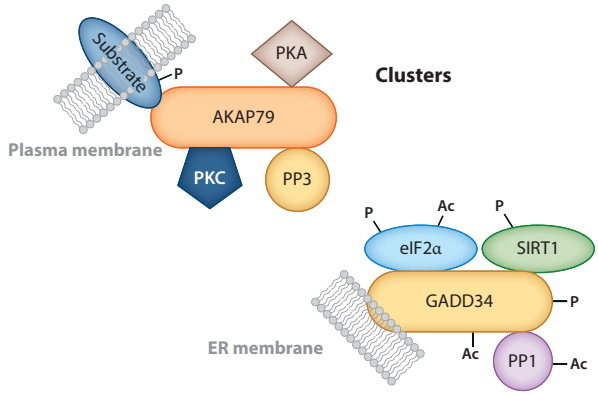

Figure 6

Protein serine/threonine phosphatases in signaling clusters. PKA-anchoring protein AKAP79 (*upper left*) recruits two distinct kinases, PKA and PKC, to tether them to transmembrane proteins that are potential substrates. AKAP79 recruits PP3, and possibly also PP1, to enable kinase-phosphatase cross talk that controls signaling by cell surface receptors and ion channels. Another recently identified cluster (*lower right*) involves the ER membrane–bound PP1 regulator GADD34. Scaffolding of PP1α and its phosphoprotein substrate eIF2α by GADD34 accounts for the substrate selectivity of this eIF2α phosphatase complex. In addition, acetylation covalently modifies PP1, GADD34, and eIF2α on one or more lysines, and acetylation of eIF2α determines the rate of eIF2α dephosphorylation. The GADD34/PP1 complex recruits another phosphoprotein substrate, eIF2α deacetylase SIRT1. Data suggest that dephosphorylation and activation of SIRT1 result in deacetylation of eIF2α. This in turn enhances eIF2α dephosphorylation by GADD34-bound PP1, creating a signaling hub that coordinates distinct posttranslational modifications. Abbreviations: Ac, acetyl; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage–induced transcript 34 protein; P, phosphate; PKA, protein kinase A; PKC, protein kinase C; PP1, protein phosphatase 1.

substrates (242). The SIRT1 acetylome likely represents only a fraction of all acetylated proteins because cells express other sirtuins and HDACs that have both nuclear and non-nuclear substrates. This information and the numerous acetylations on PPPs and their regulators documented by PhosphoSitePlus point to coordination between protein acetylation and phosphorylation in PPP signaling.

3.4. Ubiquitination in Control of PPPs

Protein ubiquitination is a reversible PTM, in many ways like protein phosphorylation and acetylation (reviewed in 243–245). Although considered primarily as the PTM that initiates proteosome-mediated degradation via polyubiquitination, protein ubiquitination represents a complex system for modifying protein function. There are multiple linkages (e.g., K48, K63, K11) as well as mono- and polyubiquitination with different branching patterns. The human ubiquitin modification system (243) contains more than 800 E3 ligases that are counteracted by approximately 90 deubiquitinases. The relative imbalance in the number of opposing enzymes recalls the situation with kinases and phosphatases. Although our understanding of the ubiquitinated proteome is not as advanced as that of the phosphoproteome, the complexity of the ubiquitin modifying system is already evident, as more than 1,000 proteins are involved in determining the ubiquitination state of more than 5,000 proteins at tens of thousands of sites (246–249). Databases such as PhosphoSitePlus (**https://www.phosphosite.org**) and mUbiSiDa (250) provide results for more than 35,000 ubiquitinated proteins and nearly 110,000 ubiquitinated sites in mammalian cells,

making this PTM as prevalent as phosphorylation. Thus, ubiquitination needs to be considered as important as phosphorylation in controlling protein function and cell physiology (251).

Ubiquitination collaborates with phosphorylation to control the half-life of many cellular proteins. There are multiple connections for cross talk between phosphorylation and ubiquitination. These include phosphorylation of E3 ligases, resulting in their increased activity or altered substrate specificity (252–254). Phosphorylation of protein substrates generates a recognition site or "degron" for reaction with E3 ligases, and an F-box domain that specifically recognizes phosphodegrons characterizes the SCF family of RING-finger E3 ligases. Ubiquitin regulation of PPP phosphatases is seen in recent work on the phosphodegron-dependent E3 ligase β-TRCP in modulating the PP1 regulator CReP/PPP1R15B and subsequently controlling eIF2α phosphorylation (255). These connections imply that phosphorylation of CReP enhances its ubiquitination by βTCRP to promote its rapid turnover, but the precise mechanisms have not been investigated. A structural relative of CReP, GADD34/PPP1R15A, also undergoes rapid degradation by the ubiquitin-proteosome system (UPS) (256). Numerous studies have shown that, following stress recovery, cells actively degrade GADD34 to the low, almost undetectable levels seen in healthy cells. Other studies (198) showed that phosphorylation of GADD34 (Tyr276) enhances its degradation by the UPS. In another example, PP1α binds the ligand-binding domain of the androgen receptor and reduces its ubiquitination and degradation by the UPS (257). Conversely, PP1γ1 binds the TRAF6 E3 ligase to enhance its autoubiquitination (258).

The list of ubiquitin E3 ligases that modify PP2A subunits continues to grow. For example, Cullin3 (259), NOSIP (260), EDD E3 ligase (261), and MID1 (262) all reportedly catalyze ubiquitination of the PP2A catalytic subunit. Paradoxically, PME-1, which hydrolyzes the C-terminal Leu methyl ester in the PP2A catalytic subunit, protects the catalytic subunit from ubiquitination (263). By contrast, MID1 (264, 265) and EDD (266) ubiquitinate alpha4, the ubiquitin-binding protein that binds the PP2A catalytic subunit to promote its ubiquitination (267). This creates a feed-forward loop of polyubiquitination that downregulates PP2A levels. Interestingly, alpha4 also associates with PP4 and PP6, but it does not seem to reduce the cellular levels of these proteins. Other E3 ligases modify PP2A subunits to regulate their turnover. The CRL4-DACF1 ubiquitin E3 ligase regulates UPS-mediated degradation of the PP2A A subunit (268), and cullin3 collaborates with Kelch-like 15, an E3 ligase adapter, to enhance proteasomal degradation of the B′β subunit (269).

Other PPPs are ubiquitinated and degraded by the proteasome. TRAF3 binds PP3 B subunit to enhance degradation of the PP3 holoenzyme (270). SCF-Cdc4 ubiquitin ligase degrades the phosphorylated and inactive form of the PP3 inhibitor protein RCN1 (271). Remarkably, as discussed above for HDAC3, binding of PP4R1 inhibits polyubiquitination of the TRAF2 and TRAF6 E3 ligases (272). Finally, recent studies showed that phosphorylation (activate) and ubiquitination (inactivate) of PP5 reciprocally control PP5 (273). Overall, these data point to numerous links among protein phosphorylation, the actions of PPPs, and E3 ligases and deubiquitinases that modify levels of proteins via the UPS.

4. COMMUNICATIONS OF PPPs IN NETWORKS, CASCADES, AND CLOUDS

4.1. PPPs in Assemblies and Networks

Complex networks of enzymes catalyze the PTMs of proteins to coordinate and balance signaling processes to control cell physiology. We anticipate that new PPP networks will be mapped by identifying new SLiMs and new interactions of PPPs with substrates and regulators and by

combining with other efforts to define protein-interaction domains in PPPs (274). Interconnections between PPPs and protein-modifying enzymes, such as deacetylases and E3 ligases, will expose new feed-forward or feedback loops to control the signals in these networks (95). Coordinating the opposing actions of kinases and PPPs also generates bistable switches that set the thresholds for signal initiation and dictate the directional flow of signals. There are numerous examples of cellular complexes that contain both phosphatases and kinases (275–277). These complexes provide for increased efficiency in signal transduction by concentrating the enzymes for modification together, but this also creates conflict by juxtaposing kinases and phosphatases. Intricate regulation of these complexes is necessary to produce discernable signals as either binary or graded outputs.

AKAP79 (A kinase–anchoring protein 79) anchors together PKA and PKC as well as PP3 at plasma membranes, where the signaling complex is tethered to membrane proteins such as receptors and ion channels (**Figure 6**) (275). Association of the AKAP79:PKA:PKC:PP3 complex with the calcium channel $Ca_v1.2$ places PP3 in close proximity to the site of calcium ion influx for a rapid response in producing NFAT dephosphorylation. Association of AKAP79:PKA:PKC:PP3 with the NMDA receptor enables cAMP-mediated phosphorylation of the ligand-gated ion channel in response to long-term potentiation of neurons, whereas PP3 in this complex mediates dephosphorylation and internalization of the receptor during long-term depression. Other membrane targets such as TRPV1 bind the AKAP79 complex that promotes their phosphorylation by both PKA and PKC. AKAP79 binds PP1 (278), further expanding the signal capacity of the AKAP79 complex with two kinases and two phosphatases.

Other signaling modules connect PKA and PP1 involving the PP1 inhibitor I-1. Phosphorylation of I-1 at Thr35 by PKA generates a potent nanomolar PP1 inhibitor, and this mode of PP1 inhibition amplifies PKA signaling, which is critical for synaptic plasticity (279) and heart contractility (280). Interestingly, a heart isoform of a PKA-anchoring protein, AKAP-18, scaffolds both I-1 and PKA to enhance the rate of PKA-mediated phosphorylation of I-1 (281). A different AKAP-18 isoform (AKAP18 is alternately spliced into four isoforms) localizes to the sarcoplasmic reticulum and binds both PP1 and the regulatory protein phospholamban. The scaffolding of PKA, I-1, and PP1 promotes rapid PP1 inhibition in response to an increase in intracellular cAMP and, in turn, enhances PKA phosphorylation of AKAP-18-associated phospholamban. This signaling cluster of enzymes is critical for β-adrenergic control of cardiac contractility, such that the loss of I-1 function or a G109E mutation in I-1 results in heart failure in animals and humans, respectively (280).

Another signaling cluster with PPP components is the STRIPAK (striatin-interacting phosphatase and kinase) complex, which is dysregulated in cancer and other human diseases (282, 283). Mammalian striatins consist of striatin, SGNA (S/G2 nuclear antigen), and zinedin, all of which can associate with the PP2A AC dimer (hence, striatin has been proposed as a B‴ subunit of PP2A). Unique among PP2A B subunits, striatins function as scaffolds to recruit members of the germinal center kinase family of protein kinases, including the mammalian sterile 20-like (Mst) kinases MST3, MST4, and STK25 (284). The STRIPAK complex recruits MST3 and facilitates its dephosphorylation and inactivation by striatin-associated PP2A (285). Various studies suggest that STRIPAK complexes control vesicular trafficking, Golgi complex assembly, cell migration, and apoptosis.

Protein complexes containing PP2A operate in the circadian cycle in *Drosophila* and, more specifically, the daytime dephosphorylation of the circadian oscillator protein CLOCK (CLK) (286). Phosphorylation of CLK, which is enhanced in the evening, inhibits transcriptional activity and promotes degradation of the CLK protein. However, during the daytime, CLK is mostly dephosphorylated and active as a transcription factor. CLK associates with two fruit fly PP2A

complexes during daytime. The fly PP2A holoenzyme containing the AC dimer bound to the B-subunit *wdb* (widerborst) binds CLK and has no impact on CLK phosphorylation but stabilizes the CLK protein. PP2A with the fly striatin *Cka* (connector of kinase to AP-1) as a subunit recruits CLK, which binds the striatin-interacting protein STRIP during daytime, and this association maintains CLK in a dephosphorylated and active state until evening. What triggers CLK recruitment by STRIPAK during daytime, how the cellular pool of AC dimers is shared between the two PP2A complexes, and what allows *Cka*-bound PP2A, but not *wdb*-bound PP2A, to dephosphorylate CLK are all questions that remain to be answered.

4.2. Coordination of PPPs with Cascades

Kinase cascades amplify signals through sequential phosphorylation steps. Researchers have discovered a growing number of phosphatase cascades in which one PPP either positively or negatively regulates the function of another PPP. For example, a PPP that dephosphorylates a phosphoinhibitor will prevent inhibition and therefore activate the PPP target of the phosphoinhibitor. An example of this two-step cascade involves dephosphorylation of I-1 by the Ca^{2+}-calmodulin-activated PP3, which is a highly efficient phosphoinhibitor-1 phosphatase. Studies in rat hippocampal slices established that calcium activation of PP3 results in rapid dephosphorylation of I-1 and, in turn, PP1 activation. This PP3:PP1 cascade (**Figure 7**), in which PP3 activates PP1, has physiological relevance in the long-term depression of hippocampal synapses (287). Mutations in PP3 catalytic A subunit have been linked to human neurodevelopmental disease associated with severe seizures (288). In another PPP cascade, PP2A promotes inhibition of PP1, i.e., PP2A activity decreases PP1 activity. This involves indirect action through phosphoinhibitors DARPP-32

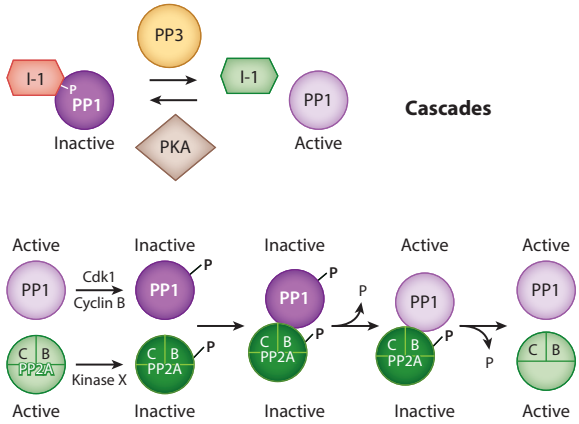

Figure 7

Cascades of PPPs. PP1 activity in cardiac muscle and hippocampal neurons is inhibited at nanomolar potency by the PKA-phosphorylated I-1. Following the influx of calcium, PP3 efficiently dephosphorylates and inactivates I-1, resulting in increased PP1 activity, producing a cascade whereby one PPP activates another to broaden the spectrum of phosphoprotein substrates that are dephosphorylated. Another PPP cascade occurs during mitosis, when the phosphorylation of PP1 catalytic subunit and PP2A B_{55} subunit by distinct mitotic kinases results in the inactivation and association of the two inactive enzymes through an RVxF motif conserved in B_{55} subunits. As mitosis progresses, cyclin B is degraded, resulting in a decrease in Cdk1 activity. PP1 then dephosphorylates itself to regain activity, and activated PP1 dephosphorylates the B_{55} subunit to reactivate PP2A. Activation of both PP1 and PP2A allows for dephosphorylation of a myriad of phosphoproteins that ensures successful exit from mitosis. Abbreviations: I-1, inhibitor-1; P, phosphate; PKA, protein kinase A; PP, protein phosphatase; PPP, protein serine/threonine phosphatase.

and I-1. These proteins contain secondary sites phosphorylated by other kinases that suppress the primary phosphorylation by PKA that converts the protein into a potent PP1 inhibitor (280, 289). These secondary phosphorylation sites are dephosphorylated by PP2A. Thus, PP2A phosphatase activity promotes PKA phosphorylation of I-1 or DARPP-32 to increase PP1 inhibition.

PP1 and PP2A are major regulators of mitosis in most eukaryotes (reviewed in 290–295). Understanding this took a long time because for years attention was directed to CDK1:cyclinB1 activation. But, abrupt activation of CDK heralds a reciprocal relationship between kinase and phosphatase activities (discussed in Section 1). Kinase inactivation of phosphatases produces bistable switching. During mitosis, both PP1 and PP2A are transiently inactivated, and reactivation of PP1 and PP2A is required for the exit from mitosis (**Figure 7**). Entry into mitosis is restricted by PP1 that inactivates the Greatwall kinase (296), thereby preventing the phosphorylation and activation of Ensa/Arpp19, a protein inhibitor of PP2A-B55. Inhibition of PP2A-B55 is an integral part of the bistable switch for robust activation of Cdk1:cyclinB1. During mitosis, PP1 function is suppressed by dual mechanisms that include Cdk1-mediated phosphorylation of the PP1 catalytic subunit at Thr320 and PP1 inhibition by I-1 (297). As cells transition from metaphase to anaphase, self-dephosphorylation of Thr320 increases PP1 activity. This increase in PP1 activity results in dephosphorylation of numerous mitotic phosphoproteins and leads to the exit from mitosis. In fission yeast that lack I-1, only one of the two PP1 inhibitory mechanisms operates; specifically, PP1 is inactivated during mitosis via phosphorylation of Thr316 (equivalent to mammalian Thr320) by the yeast Cdk1:Cyclin B complex (128).

During mitosis, two different PP2A holoenzymes (containing B55 and B56) are inactivated by phosphorylation of their B subunits, but the kinases remain unknown. The yeast B55 and B56 subunits (Cdc55 and Rts1) both contain SLiMs that bind PP1, and these sequences are conserved in human B subunits. Interestingly, PP1 is preferentially recruited to the phosphorylated PP2A:B55 heterotrimer, not the phosphorylated PP2A:B56 because this B subunit contains a phosphoserine (pSer378) between two PP1-binding SLiMs. As mitosis proceeds and cyclin B is degraded, Cdk1 activity wanes, and self-dephosphorylation of Thr316 activates PP1, which then dephosphorylates the inhibitory phosphorylation site in B55 to reactivate the PP2A:B55 holoenzyme (**Figure 7**). Even more remarkable, activated PP2A:B55 then dephosphorylates pSer378 near the PP2A:B56 SLiM, allowing recruitment of PP1 to PP2A:B56. PP1-mediated dephosphorylation of the inhibitory phosphosite on B56 then leads to full activation of the PP2A:B56 phosphatase and exit from mitosis. The end result is that both PP1 and PP2A holoenzymes are active and dephosphorylate hundreds of phosphorylation sites on mitotic phosphoproteins (298–301) to exit mitosis.

Repo-Man, which was first identified as a PP1-binding protein by SILAC proteomics, recruits PP1 to chromatin during mitosis (121). Studies searching for potential Repo-Man-interacting proteins identified PP2A:B56γ (302). These exciting studies by Qian et al. (303) deciphered a novel PPP switch where the functions of the two phosphatases associated with Repo-Man are carefully choreographed during mitosis. Specifically, Cdk1 binds Repo-Man and catalyzes its phosphorylation near the two PPP-binding sites during mitosis. Phosphorylation near the RVTF PP1-binding site displaces PP1 from Repo-Man, whereas phosphorylation adjacent to the LSPI PP2A-binding site enhances recruitment of PP2A:B56. As Cdk1 activity drops owing to cyclin B1 degradation, phosphorylation sites in Repo-Man become dephosphorylated, although the phosphatase(s) responsible has yet to be identified. This diminishes PP2A:B56 association and increases PP1 association with Repo-Man. The PP1:Repo-Man complex is targeted to chromosomes to dephosphorylate and inactivate Aurora B. Phosphorylation and dephosphorylation of PPP catalytic and regulatory subunits, as discussed above, are fundamental mechanisms for the timing, coordination, and targeting of phosphatase activities.

4.3. Organization of PPP Clouds

We have highlighted the capacity of PPPs to communicate with each other using clusters, cascades, and switches that orchestrate their collective functions. This cross talk should generate PPP networks that coordinate multiple aspects of cell physiology to determine health and disease. The interconnectivity of signaling networks creates larger "clouds" or mega-networks, which is a bioinformatics concept (adopted from 304) that describes a broad and highly interconnected network created by linking a host of smaller sub-networks. Computational modeling suggests that such clouds take at least two forms, stratus-type and cumulus-type clouds. In a stratus-type cloud the PPPs within the network communicate with each other in a highly dynamic fashion, with no apparent node of functional dominance for any single PPP. Recent studies examined the transition that stem cells make from self-renewal to terminal differentiation as keratinocytes (305). Transcriptomic and proteomic data from differentiating human keratinocytes were combined with small interfering RNA (siRNA)-mediated knockdown to identify phosphatases that catalyze the dephosphorylation events that are pivotal for cell commitment. A total of seven phosphatases specific for pSer/pThr or pTyr, or with dual specificity, were identified as required for commitment (**Figure 8**). These phosphatases regulate MAP kinase signaling and activator protein-1 (AP1)-mediated gene transcription. Mathematical modeling that deciphered the functional interactions within this autoregulatory network suggested extensive positive and negative interactions that dictated the expression of each phosphatase over the time course of differentiation. In a highly simplified view, interactions between these phosphatases during early stages of differentiation were few in number and mostly negative, but as the commitment process proceeded (at 4 h), the interactions grew more numerous and were mostly positive before eventually returning to a state with fewer and mostly negative interactions (seen after 12 h). Nevertheless, these final interactions were quite different from those seen at the start of the commitment process. These data highlight the complex and dynamic reshaping of this phosphatase network as well as the extensive cross talk between phosphatases during keratinocyte differentiation. We propose that this signaling network fulfills the criteria of a stratus-type phosphatase cloud.

By contrast, the phosphatase network controlling mitosis is very different, as noted above, with dominance of PP1 and PP2A and multiple distinct PPP complexes formed by PP1 bound to Repo-Man, MYPT1, SDS22, and other proteins as well as PP2A bound to Shugoshin, BubR1, and Repo-Man. These multiple PPP complexes control various transition points during mitosis in mammalian cells. Indeed, there is accumulating evidence that several other PPPs, including PP3 (306, 307), PP4 (308), PP5 (309), and PP6 (44), play roles in mitosis. St-Denis et al. (310) utilized affinity purification and proximity-based interaction proteomics followed by an siRNA screen targeting multiple families of protein phosphatases and their known regulators to establish the phenotypic and interaction profiles of human phosphatases as mitotic regulators. The proteomics screen observed a total of 1,335 high-confidence interactions among the 140 proteins containing phosphatase domains, suggesting extensive contacts or communication between phosphatases that likely control many different cell functions. The siRNA screen identified 48 candidate mitotic phosphatases, and phenotypic analyses showed that 45 of these regulated spindle assembly and 27 modulated mitotic progression. As anticipated, PP1 and PP2A complexes were dominant among the mitotic regulators, but unexpectedly, 13 of the candidate regulators were protein Tyr phosphatases, including dual specificity phosphatases and receptor Tyr phosphatases. Although these studies did not address substrates or transient interactions, they highlight the complexity of the phosphatase network that regulates mitosis in mammalian cells. Thus, even with a narrow focus on PPPs, the signaling cloud for mitosis is very different from that described for differentiation. The major role that PP1 and PP2A play in controlling mitosis in mammalian cells predicts a

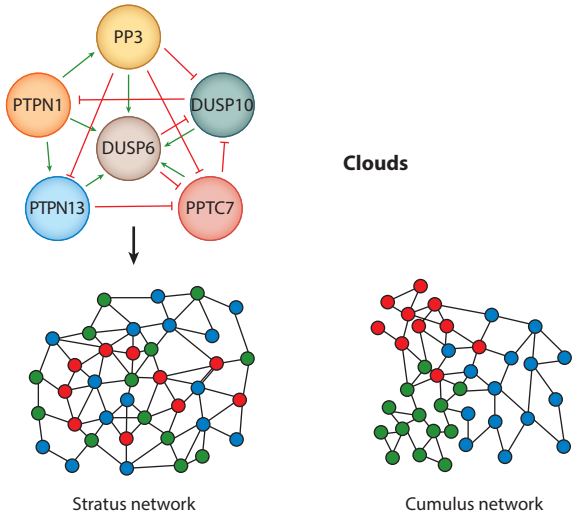

Figure 8

Stratus-type and cumulus-type cloud networks of protein serine/threonine phosphatases (PPPs). Signaling networks can contain multiple phosphatases. One network involved in the commitment of stem cells into keratinocytes contains protein phosphatase 3 (PP3) as well as a PPM family member (PPTC7), two phosphotyrosines (PTPN1 and PTPN13), and two dual-specificity phosphatases (DUSP6 and DUSP10). The simplified schematic shows a snapshot of positive and negative interactions between component phosphatases in this network, although these interactions may occur in different time frames during the differentiation process. We speculate that this resembles a stratus-type cloud network, similar to that proposed by computational studies of protein dynamics, in which the physiological event relies on signaling by multiple phosphatases with no single phosphatase completely dominating this network. This also predicts the presence of other cumulus-type clouds or networks that are comprised of several subnetworks or communities in which there may be a preponderance of a specific PPP, such as among the many PP1 and PP2A holoenzymes that control events in mitosis. Cumulus cloud networks allow for some autonomy among individual subnetworks, such that distinct PP1 (or PP2A) complexes may communicate via shared regulatory mechanisms such as covalent modifications within or near short linear motifs that promote a dynamic exchange of regulatory subunits or alter subcellular localization or substrate recognition by newly formed holoenzymes. There is accumulating evidence that these networks are not static but can be remodeled or even interconverted by physiological and pathological stimuli. These networks or clouds function in noise filtering, control signal flexibility, and adapt to change the directionality and flux of signaling pathways, thereby successfully executing complex physiological events. Bottom panels adapted from Reference 304 with permission.

concentration of functions driven by these enzymes or enzyme complexes. Thus, the PPP cloud controlling mitosis will likely have a cumulus-type structure with foci or areas of connectivity representing the processes controlled by one PPP, e.g., PP1 or PP2A, albeit functioning in multiple molecular complexes. Individually, these two types of clouds are predicted to direct information flow quite differently. The cumulus-type cloud possesses significantly more three-dimensional networks than does the stratus-type cloud and thus is both more powerful and more efficient in distributing signals. Nevertheless, these two types of information network clouds provide tremendous flexibility for cell signaling. Understanding the components and connectivity of PPP clouds will be critical to evaluate how age, stress, diet, and other factors both negatively and positively impact PPP signaling. That knowledge will allow for better prediction of the benefits and liabilities of future therapies that target PPPs.

5. CONCLUDING REMARKS

This review is focused on PPPs, only one of the three protein Ser/Thr phosphatase families. In the more than 60 years since phosphorylase phosphatase was first identified (2), extensive research on the structure, function, and regulation of phosphoprotein phosphatases has produced more than 100,000 publications. The rapid pace of advance and breadth of current phosphatase research is reflected in the nearly 200 reviews, opinions, and commentaries that have been published in the past 5 years. The international phosphatase community gathers to share new developments at regular biannual conferences held in the United States, Europe, and Japan. Nearly half the participants at these meetings are new to the field, fueling expansion while interjecting novelty and innovation. The July 2018 FASEB Summer Research Conference on Protein Phosphatases will be the 15th in this series and celebrates the 30th anniversary of the discovery of protein Tyr phosphatases. The EMBO Conference Series on Europhosphatases held in Paris, France, in 2017 was the 14th in this series, and the 13th International Conference of Protein Phosphatases will be held in Tokyo, Japan, in 2018. With such proliferation of information, a single comprehensive review on protein phosphatase research would be near impossible. We had to make some choices and elected to examine the discovery and utility of SLiMs, which are present in substrates and regulators and have been used to identify and study hundreds of PPP interactors. In addition, we highlight the continued growth of the ultradeep phosphoproteome (27) to increase awareness about how protein phosphorylation regulates PPPs. We discuss studies that suggest the modulation of PPP functions by acetylation and ubiquitination. Last but not least, we point to the complexity of cell regulation that involves PPP networks that coordinate and integrate signals to effectively execute complex biological events such as cell commitment or mitosis. Expertise in computational biology and bioinformatics will be needed to better understand the functions and regulation of PPP phosphatases. By focusing our discussion, we were unable to include other developments in the field or recognize the contributions of many fellow investigators. We encourage readers to see other recent reviews to more fully appreciate the astonishing progress of this vibrant and exciting research field.

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