Mechanisms regulating phosphatase specificity and the removal of individual phosphorylation sites during mitotic exit

Samuel Rogers [1)], *Rachael McCloy* [1)], *D. Neil Watkins* [1)2)3)] *and Andrew Burgess* [1)2)*]

Entry into mitosis is driven by the activity of kinases, which phosphorylate over 7000 proteins on multiple sites. For cells to exit mitosis and segregate their genome correctly, these phosphorylations must be removed in a specific temporal order. This raises a critical and important question: how are specific phosphorylation sites on an individual protein removed? Traditionally, the temporal order of dephosphorylation was attributed to decreasing kinase activity. However, recent evidence in human cells has identified unique patterns of dephosphorylation during mammalian mitotic exit that cannot be fully explained by the loss of kinase activity. This suggests that specificity is determined in part by phosphatases. In this review, we explore how the physicochemical properties of an individual phosphosite and its surrounding amino acids can affect interactions with a phosphatase. These positive and negative interactions in turn help determine the specific pattern of dephosphorylation required for correct mitotic exit.

Keywords:
Cdk1; kinase; mitotic exit; phosphatase; phosphorylation; PP2A; PP1.

Introduction

Entry into mitosis involves the physical compaction of the duplicated genome into pairs of chromatids known as chromosomes, dissolution of the nuclear envelope [1], reorganisation of the actin cytoskeleton to induce cell rounding [2] and formation of the mitotic spindle [3]. The mid-point of mitosis (metaphase) is characterised by the successful bi-orientated attachment of spindle microtubules to the kinetochores, large multi-protein structures located at the centromere of each sister chromatid. Successful attachment satisfies the major mitotic checkpoint, the spindle assembly checkpoint, which in turn marks the start of mitotic exit [4]. During mitotic exit, sister chromatids are pulled towards opposite poles of the cell by the mitotic spindle, followed by the subsequent equatorial cleavage of the cell membrane producing two distinct but identical daughter cells [5]. During mitotic entry transcription, and to a lesser extent translation are repressed [6], consequently, post-translation modifications are largely responsible for driving the physical events of mitosis. Of these, phosphorylation is the most abundant and arguably the most important, with over 32 000 phosphorylation events occurring in mitosis [7]. This extensive network of phosphorylation events is directly and indirectly driven primarily by the activity of cyclin-dependent kinase 1 (Cdk1) [8]. Once cells successfully attach all chromosomes to the mitotic spindle in metaphase, the activity of Cdk1 is no longer required. Consequently, as cells exit mitosis, Cdk1 is inhibited, and all of the phosphorylation events it made are removed. These two events are driven by protein degradation and protein phosphatases, respectively. Briefly, at the metaphase to anaphase transition, the regulatory subunit of Cdk1, Cyclin B1, is targeted by the APC^{cdc20} for degradation by the proteasome, resulting in a loss of Cdk1 activity [9,10]. In addition to Cyclin B1, the best current estimates predict that approximately 170 proteins are degraded during mitotic exit in mammalian cells [11]. In yeast, this is potentially higher with up to 10–20% of proteins being degraded during exit [12]. This degradation is critical for preventing the reactivation of the spindle assembly checkpoint [13], thereby ensuring that exit occurs in only one direction [14] and is irreversible [15]. For more information on the roles of protein degradation during mitotic exit, please see [16–18].

While protein degradation is critical for correctly exiting from mitosis, it is unable to remove all of the ~32 000 phosphorylation events that are made in mitosis [7,19]. Current estimates indicate that only a small percentage of proteins are degraded during mitotic exit (~2.5% in humans and up to 20% in yeast) [11]. Therefore, the vast majority must be removed by phosphatases. In human cells, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) superfamilies have been implicated as the major Cdk1 counter acting phosphatase

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[1)] The Kinghorn Cancer Center, Garvan Institute of Medical Research, Darlinghurst, NSW 2010, Australia
[2)] St. Vincent's Clinical School, Faculty of Medicine, UNSW, Darlinghurst, NSW, Australia
[3)] Department of Thoracic Medicine, St Vincent's Hospital, Darlinghurst, NSW 2010, Australia

***Corresponding author:**
Andrew Burgess
E-mail: a.burgess@garvan.org.au

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[20]. Both PP2A and PP1 exist as multimeric protein complexes, with 70–200 potential configurations respectively, which help provide specificity for these phosphatases. This specificity is critical, as the removal of mitotic phosphorylation events must proceed in a temporal co-ordinated manner to ensure that the events of mitotic exit occur correctly and each daughter cell receives an identical copy of the genome. This poses an important question, what are the mechanisms that determine how and when PP1 and PP2A decide to remove a specific phosphorylation site during mitotic exit?

The dephosphorylation of mitotic substrates: Lessons from mass spectrometry

In the past decade, advancements in mass spectrometry-based techniques have led to a number of high-resolution studies analysing the global phosphoproteomic changes during mitosis. These studies range from yeast to human and incorporate multiple conditions and comparisons. For example, studies in budding yeast suggest that temporal protein dephosphorylation during mitotic exit can be broadly grouped into four distinct groups: early, intermediate, late and stable [12,21]. Importantly, proteins that are associated with the early mitotic exit events, such as silencing of the spindle assembly checkpoint, spindle elongation, chromosome segregation and central spindle formation, are all dephosphorylated first. Proteins involved in regulating subsequent events of telophase/cytokinesis, such as mRNA processing, chromatin decondensation, and replication origin firing, are not dephosphorylated until the later stages of mitotic exit, highlighting the important role that dephosphorylation plays in the temporal ordering of mitotic exit events. We also recently observed similar trends in human cells [22], suggesting that the temporal dephosphorylation of proteins is evolutionarily conserved (Figure 1).

One draw back of these global patterns of dephosphorylation is that they are based on the average of all phosphorylation sites on a protein. Consequently, they do not take into account the contributions of individual phosphorylation sites. Furthermore, by taking the average, there is the assumption that all sites within a protein are dephosphorylated at a similar rate. However, during mitotic exit, significant intra-protein heterogeneity is observed on phosphosites of individual proteins, even on those that are in the early and late/stable groups (Figure 1). Consequently, dephosphorylation of one site on a protein does not guarantee that the other sites on that same protein are also dephosphorylated [22]. This mosaic pattern of intra-protein dephosphorylation during mitotic exit has also been observed by others [23,24]. The reasons for heterogeneity are still unclear, although it has been suggested that it may provide the additional capacity for regulating the events of mitotic exit by combining multiple phosphorylation sites on the same protein [25]. What is clear is that ultimately there must be mechanisms that determine the specificity of dephosphorylation at the level of the individual phosphosite. Furthermore, it also means that subcellular localisation cannot fully account for the specificity of dephosphorylation. The purpose of this review is to explore some of the potential mechanisms for this intra-protein variation in dephosphorylation during mitotic exit.

Phosphorylation: A balancing act

Phosphorylation of a protein is often used as a functional readout of kinase activity. The presence of a phosphorylation is often taken to mean that the upstream kinase is active, while loss of phosphorylation suggests that the kinase is inhibited; however,

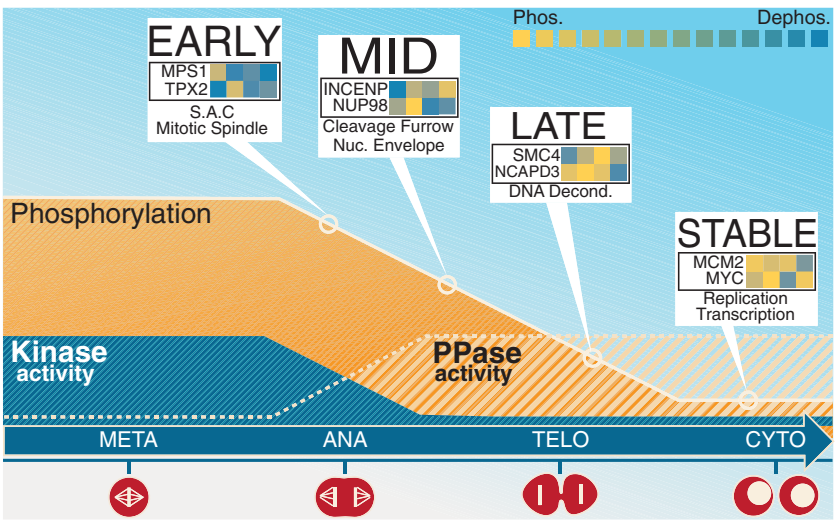

Figure 1. Diagrammatic representation of the staggered and ordered dephosphorylation of substrates during mitotic exit. Dephosphorylation of proteins broadly corresponds with proteins known to be involved in regulating key events during mitotic exit. Early events include silencing of the spindle assembly checkpoint (SAC) and mitotic spindle elongation. Mid events include formation of the cleavage furrow and reformation of the nuclear envelope. Late events include decondensation of the DNA. Within proteins at the level of individual phosphorylation sites, there is significant heterogeneity (yellow = phosphorylated and blue = dephosphorylated residue), which cannot be completely explained by decreasing kinase activity. Consequently, additional factors contribute to the specific dephosphorylation of phosphosites by phosphatases during mitotic exit.

it could also be that a phosphatase has been activated. Consequently, the presence (or absence) of a phosphorylation at any particular point in time is a balance in activity between the regulatory kinase and the counterbalancing phosphatase. Therefore, in order to fully understand the mechanisms controlling the variable dephosphorylation of specific sites within a protein, both the kinase and the phosphatase must be taken into account.

Mitotic kinases and their impact on dephosphorylation kinetics

Kinases comprise ~2% of the entire genome and are one of the most comprehensively studied class of enzymes within the proteome. During mitosis, the overwhelming majority of phosphorylation events occur on serine and threonine residues (~85% and ~15%, respectively) with less than ~1% occurring on tyrosine residues [7,19,22,26,27]. Consequently, Ser/Thr-directed kinases, such as Cdk1, Plk1, Aurora A/B, Nek2 and CK1/2, are responsible for the majority of phosphorylation events in mitosis. These kinases, which share a similar basic structure and catalytic mechanism, generate specificity for substrates in part through differences in the charge and hydrophobicity of surface residues present on each kinase [28]. As a result, each kinase displays a relatively high degree of specificity for individual phosphorylation sites based on the unique amino acids at, and surrounding, the accepting Ser/Thr [28]. This has led to numerous large-scale proteomic studies utilising small molecule inhibitors [26,29–31] and the development of numerous tools such as peptide spot library arrays for determining kinase substrates and phosphorylation motifs [32,33]. The results of these can be found in large online databases such as PhosphoSitePlus (http://www.phosphosite.org) and computational prediction tools such as ScanSite [34], Group Based Prediciton Software (GPS) [35], KinomeXplorer [36] and PSEA [37]. Consequently, there is now a plethora of information and tools available for studying the impact kinases play on substrate phosphorylation. Using these resources combined with quantitative phosphoproteomics, we recently analysed the impact that Cdk1 inhibition has on the dephosphorylation of substrates during mitotic exit. Surprisingly, despite strong inhibition of Cdk1 activity, no preferential dephosphorylation of the Cdk1 consensus motif (S/T-P-x-K/R) was observed during the early phases of mitotic exit [22,38]. This suggests that although loss of Cdk1 kinase activity is critical for mitotic exit, it is not solely responsible for determining the specific dephosphorylation of substrates. This is supported by work in yeast, which also found no significant correlation between early substrate dephosphorylation and the loss of Cdk1 activity; however, late substrates were affected [21,22]. Consequently, a kinase centric model of mitotic exit, where a gradient of decreasing kinase activity results in a stepwise decrease in phosphorylation on substrates, is not able to fully explain all of the dephosphorylation events that are observed. Therefore, additional mechanisms beyond simple alterations in kinase activity must exist to determine dephosphorylation timing and specificity, with the most obvious candidate being phosphatases.

Regulation of mitotic phosphatase activity

In recent times, several landmark publications have highlighted the important roles that phosphatases play in regulating mitosis. In yeast, Cdc14 is the major mitotic phosphatase responsible for dephosphorylating Cdk1 substrates and driving mitotic exit [39]. However, in higher eukaryotes and mammalian cells, protein phosphatases PP1 [40–43], PP2A [41,44–48] and potentially Fcp1 [49,50] have been shown to dephosphorylate substrates during mitotic exit (also reviewed in [44,51]).

For many years, it was assumed that PP1 and PP2A were constitutively active enzymes; however, it is now clear that the activities of both are suppressed concomitant with Cdk1 activity during mitosis. Failure to inhibit phosphatase activity has severe consequences, with cells undergoing a highly aberrant mitosis with multiple defects [46,52,53]. The inhibition of both PP1 and PP2A is in part mediated by the binding of several small unstructured, heat-stable phosphoproteins, which potently inhibit PP1 and PP2A during mitosis. The benefits that this lack of structure has on inhibiting phosphatases will be discussed in later stages of this review. During mitotic entry, alpha-endosulfine (ENSA) and the highly related Arpp19 are phosphorylated by Mastl (also known as Greatwall kinase, Gwl), PKA and Cdk1 [54–57]. These phosphorylation events greatly enhance the binding of ENSA/Arpp19 to PP2A-B55 blocking the active site of the phosphatase. PP2A-B55 then slowly removes the phosphorylation on ENSA, allowing its subsequent release [58]. Of note, phosphorylated ENSA is in stoichiometric excess to PP2A-B55 [58], which may explain the conflicting report that Fcp1 dephosphorylates ENSA [59], with the remaining free pool of ENSA potentially dephosphorylated by Fcp1. What is clear is that phosphorylated ENSA is a potent competitive inhibitor of PP2A activity. This mode of inhibition appears to hold true for the other small inhibitory proteins with phosphorylation of Inhibitor 1 (I-1)/DARPP-32 [41], and Inhibitor 2 (I-2) by PKA and Cdk1, respectively, necessary for inhibition of PP1 [60–62]. Similarly, Cdk1 phosphorylation of Bod1 on T95 results in the inhibition of PP2A-B56, which is required for correct kinetochore-microtubule attachments during mitosis [63].

In addition to inhibitory proteins that directly interact with phosphatases, proteins with phospho-binding domains provide an additional layer of regulation by competing with phosphatases for binding to phosphorylation sites. Examples of key serine/threonine phospho-binding domains include 14-3-3 proteins, WD40 repeats, forkhead-associated, Polo-box domains, Breast Cancer type 1 (BRCA1) carboxy-terminal and WW domains [64]. The protein Pin is a classic example of a mitotic exit regulator, which binds to phospho-sites through its WW domain. The impact of Pin1 on dephosphorylation during mitotic exit will be discussed in more detail later in this review.

A final layer of regulation exists for both PP1 and PP2A, as both form multimeric protein complexes. There are several excellent reviews on all of the various subunits and how they regulate mitotic phosphatase activity, for PP1, see [65,66] and PP2A [67,68]. Briefly, PP1 consists of a catalytic core (C-subunit), which can bind with >200 interacting proteins, thereby creating over 650 potentially distinct complexes [65,66]. These regulatory subunits often bind to a site allosteric to the PP1 active site through docking motifs, with the amino acid motif RVxF found in over 90% of all PP1 binding partners to date [69]. Binding is often supported by several other ancillary motifs, such as G/SILK, which co-operate with the RVxF motif to promote binding. PP2A exists as a trimeric complex consisting of a catalytic (C-subunit), a scaffolding (A subunit) and a

regulatory (B-subunit). In the majority of cases, the C-subunit binds to the A-subunit to form a stable base complex, which then recruits a regulatory B-subunit providing specificity. The B-subunit contains four distinct subfamilies (B, B', B" and B"') encoded by 15 distinct genes, with over 26 potential isoforms and splice variants [67]. The B and B' subunits (also known as B55 and B56, respectively) are the only subunits associated with regulating mitotic exit in higher eukaryotes and human cells [44]. In addition, recent evidence suggests that trimeric PP2A complexes can also interact with addition proteins that regulate PP2A by restricting it to a specific subcellular localisation. For example, PP2A-B56 binds to BubR1 phosphorylated on S670 and S676, which creates an LSPI binding motif. The binding of BubR1 promotes the localisation of PP2A-B56 to kinetochores where it acts to dephosphorylate Aurora B substrates [70,71] and ensures correct chromosome alignment at the metaphase plate [72]. Thus, PP2A-B56 binds to BubR1 phosphorylated on S670/676, but does not appear to dephosphorylate these sites [22], as it would prevent PP2A from reaching the kinetochore. Interestingly, the LSPI motif is also present in the PP1 interacting protein Repo-Man, with phosphorylation of the serine in this motif (S591) again promoting PP2A-B56 binding. However, this binding promotes the subsequent dephosphorylation of S893 on Repo-Man [73]. These examples highlight the issue that binding of a phosphatase to a protein does not guarantee that it is a substrate of the phosphatase. Consequently, a major gap in our understanding of how and which PP1 and PP2A complexes regulate mitotic exit is due to the limited number of validated phosphatase–substrate relationships. To date, examples of PP1 substrates include ezrin/moesin [42,43], Kif18 [74,75], Scp105 [76] and kinesin's [77]. Substrates of PP2A include securin [78,79], Aurora A [80], with ENSA specifically dephosphorylated by PP2A-B55 [58], and Kif4a [81], Knl1 [82] and the aforementioned Repo-Man [73] dephosphorylated by PP2A-B56. This raises a critical issue, which is how are these phosphatases directed towards specific phosphorylation sites on a protein?

Mechanisms for phosphatase specificity and the global trends of dephosphorylation

One potential answer for how PP1/PP2A specifically target and dephosphorylate individual sites on a protein is that just like kinases, substrates contain distinct motifs that promote or inhibit interactions. Traditionally, this model has been difficult to prove as both enzymes show varying abilities to dephosphorylate short peptide sequences *in vitro*. For example, PP2A is up to 100-fold more active as a trimeric complex compared with the catalytic subunit alone [83], while PP1 often has difficulty binding short peptide sequences by itself, because of the dependence on regulatory subunits for strong substrate binding [65]. These factors have limited our ability to accurately measure the contribution of neighbouring amino acids in the manner that kinases have.

However, we recently demonstrated that specific amino acids at and surrounding the phosphosite correlated with dephosphorylation during early mitotic exit [22,38]. These dephosphorylation patterns were evenly distributed across all major kinases, indicating that changes in kinase activity could not fully explain the observations. This suggests that local amino acids might impact phosphatase specificity. In support, TP sites were more commonly dephosphorylated compared with SP sites during mitotic exit (1:0.85 ratio) [22]. This enrichment occurred despite the significantly lower relative abundance within the proteome of proline-directed threonine (TP) residues compared to serine (SP) residues (1:1.5 for TP versus SP respectively). This preferential dephosphorylation of lower abundance TP sites during mitotic exit has also been reported by others in mammalian cells [24,28]. Interestingly, this situation is reversed in budding yeast, where SP residues are preferentially dephosphorylated by the Cdc14 phosphatase [12], which may explain the divergent roles of Cdc14 between organisms. In yeast, Cdc14 primarily drives mitotic exit, whereas in higher eukaryotes, the role of Cdc14 is less clear [84].

The second observation was that acidic residues (D, E) upstream (right) and to a lesser extent downstream of any (S or T) phosphorylation site correlated with stable phosphorylation sites, indicating that acidic residues negatively impact dephosphorylation. Likewise, an additional proline (P) upstream (+2) of a TP (TPP motif) and to lesser extent SP phosphosite (SPP motif) was also associated with significantly less dephosphorylation. Switching the proline at the +2 position for a small non-polar side chain amino acid (TPG/A/V motif) appears to alleviate the negative impact and promote dephosphorylation during mitotic exit [22]. Taken together, it suggests that the local amino acid environment can have a strong negative impact on the ability of phosphatases to dephosphorylate a specific site. The following sections will discuss the potential mechanisms for how phosphatase specificity produces these experimental observations.

The catalytic clefts of PP1 and PP2A and the preference for phospho-threonine residues

The preferential dephosphorylation of threonine over serine phosphosites in higher eukaryotes has been reported by several groups [24,85], and we recently showed that this extends to proline-directed sites (TP over SP). This suggests that there is some common intrinsic preference built into the catalytic core of PP1/PP2A that confers this favouritism. In support, the catalytic sites for both PP1 and PP2A contain similar tertiary structures, where the key residues essential for catalysis of phosphosites are reasonably well conserved. These essential residues are at the centre of both active sites and bind to two divalent metal ions. For PP1, these are iron and zinc [86,87], while for PP2A, it is most likely manganese [88]. These metal centres modify the geometry of the substrate phosphate group upon phosphatase binding. Such changes enable the movement of the oxygen atoms around the central phosphorous, which in turn ensures that the substrate phosphate can bind tightly to the metal centres and increases the overall reactivity of the phosphate group [86]. This aids the tight association between the active site and the substrate protein to ensure efficient removal of the phosphate from the serine or threonine residue. Interestingly, this tight association between the metal complex and the phosphorylation may explain why protein inhibitors such as inhibitor-1 and ENSA bind with much higher

affinity when phosphorylated [41,56–58,89] and may also explain the presence of the phosphate groups in some small molecule phosphatase inhibitors.

An important question is how this general similarity in the activity sites of PP1 and PP2A confers specificity for phosphorylated threonine over serine residues? One potential explanation is that threonine residues fit more favourably into the active site of PP1 and PP2A compared with serine. In support, crystal structures of PP1 and PP2A indicate that both contain conserved hydrophobic residues in the regions adjacent to the metal ions in the active site. These regions correspond to where the side chain of phosphorylated serine and threonine residues reside [86]. Threonine differs from serine in that it is slightly larger and more hydrophobic [90]; therefore, threonine could form more energetically favourable contacts with these hydrophobic residues and contribute to a stronger phosphatase : phosphate complex compared with serine (Figure 2A). This small increase in affinity could explain the observed preference that PP1/PP2A has for threonine over serine residues during mitosis.

Surface surrounding PP1/PP2A active site and its impact on substrate interactions

Our observation that acidic residues surrounding a phosphosite negatively impacted dephosphorylation could also be explained by common structural elements present in PP1/PP2A. Specifically, amino acids adjacent to the phosphorylation site on a substrate could interact positively or negatively with the surface adjoining the catalytic site of PP1 and PP2A, thereby promoting or inhibiting the association between PP1/PP2A and substrates. Consistent with this idea, the active site of both PP1 and PP2A is at the base of a shallow surface groove. While the surface

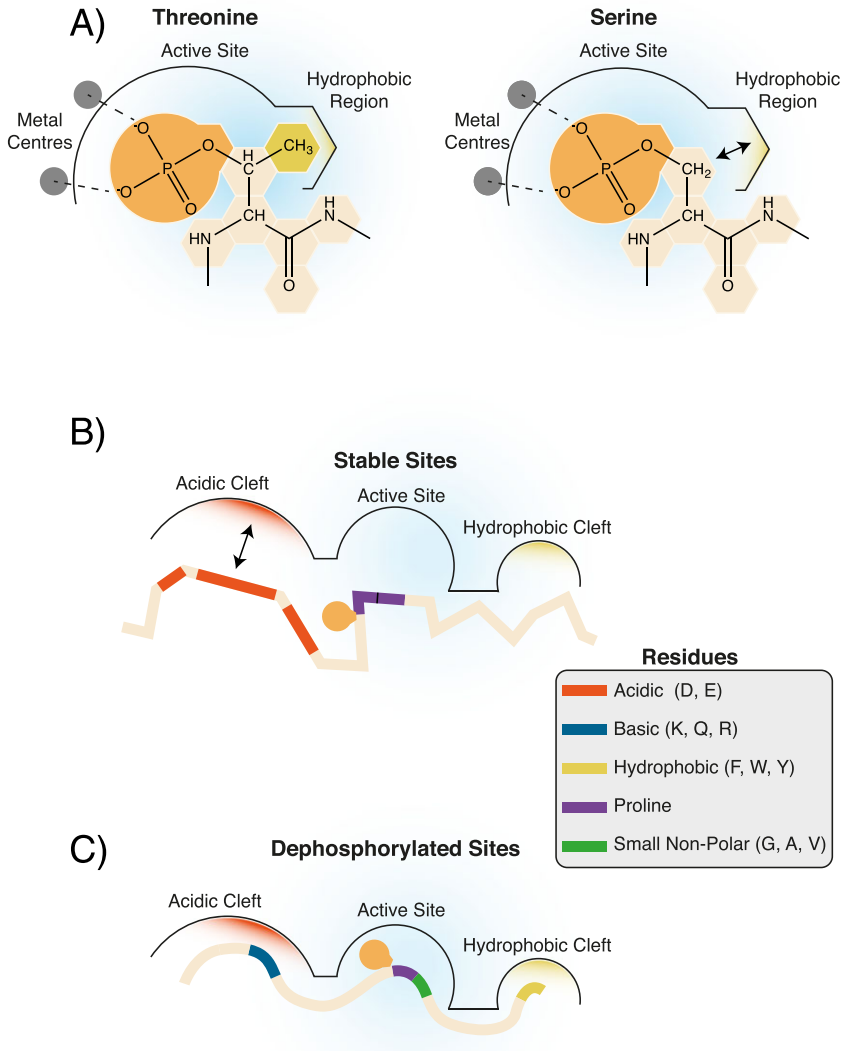

Figure 2. **A:** Potential mechanism for the observed preferential dephosphorylation of threonine over serine residues. Threonine is slightly larger and more hydrophobic, which could aid favourable interactions with the hydrophobic region within the catalytic cleft of PP1 and PP2A. **B** and **C**: Enrichment of specific amino acids adjacent to the phosphorylation site in substrates and their interaction with the surface surrounding the active site of PP1/PP2A provide an explanation for the observed enrichment of these residues in stable (B) and dephosphorylated (C) phosphosites. In addition, the conformational structure of the substrate may also impact on the ability to bind efficiently with the phosphatase with rigid structures (B) less likely to interact in comparison with disordered, flexible (C) regions.

surrounding the catalytic core is composed of a shallow Y-shaped groove with acidic, C-terminal and hydrophobic regions [91,92]. Consequently, acidic residues adjacent to the phosphorylation site could create unfavourable interaction between the substrate and the corresponding acidic residues on the phosphatase (Figure 2B). The repulsion between these residues could reduce the affinity of substrate : phosphatase binding and reduce the rate of dephosphorylation. This could explain why acidic residues were correlated with stable phosphosites [22], although it should be noted that for non-proline-directed sites, this preference may result from the continued activity of kinases such as CK2 and Plk1, which have a preference for acidic residues up and downstream of the phosphosite, respectively. However, the enrichment of acidic residues occurred even when the kinase preference was taken into account [22,38], suggesting that this negative affect does play a role. In support, basic residues (K/R), which would bind more favourably to the acidic surface of PP1/PP2A where enriched in a subset of dephosphorylated phosphopeptides (Figure 2C).

It should also be noted that the binding of regulatory subunits for both PP1 and PP2A can also impinge on the local area near the catalytic site, thereby altering the interaction between substrates and phosphatase. For example, the regulatory subunit spinophilin binds to PP1 occupying the C-terminal cleft. This impedes substrates that utilise this site from binding to PP1 [93] and conversely likely favours binding with substrates that possess hydrophobic or basic elements. A similar mechanism is observed with MYPT1 binding to PP1. This binding extends the acidic and hydrophobic cleft of the PP1 catalytic core, creating a surface more compatible with binding to, and dephosphorylation of myosin (~10-fold greater activity), while simultaneously reducing the affinity for other substrates [94]. In summary, it is clear that the local amino acids surrounding a phosphosite can impact the ability of PP1/PP2A to bind to and therefore dephosphorylate a substrate.

The geometry of a phosphosite and its effects on dephosphorylation

The impact of proline in the +1 position appears to play an important role in regulating the dephosphorylation of substrates, with proline-directed sites dephosphorylated significantly more than non-proline-directed sites during the early stages of mitotic exit [22]. Furthermore, the enrichment for acidic residues in proline-directed sites was far less pronounced, suggesting that the proline in the +1 position has a more dominant effect on phosphorylation than neighbouring acidic amino acids. One potential explanation for this is that the addition of a proline residue promotes disorder by disrupting the formation of secondary structures (α-helix and β-sheet) within proteins [95]. Interestingly, phosphorylation predominantly occurs within regions of the protein that have no regular secondary structure. The secondary structure, and in particular, the disordering effects of proline, could play a regulatory role in determining phosphatase specificity for the phosphorylation sites. Regions with no fixed structure surrounding the phosphorylation site have more potential to adopt multiple configurations, which could allow the proline-directed phosphosites to fit into the phosphatase catalytic site more easily (Figure 2C). Conversely, fixed, rigid conformations could block and restrict binding, thereby reducing the rate of dephosphorylation (Figure 2B) [96]. This appears to be the case for at least PP2A where restricting the conformation of residues adjacent to the phosphorylation sites prevented the dephosphorylation by PP2A [97,98]. While *in vitro* peptide dephosphorylation assays also show that an additional proline significantly decreases the PP2A-mediated dephosphorylation of both threonine and serine residues [83].

Notably, we found evolutionally conserved examples of this mechanism during mitotic exit. There was a clear enrichment of phosphosites that remained stable during mitotic exit, which contained an additional proline at the +2 position. In contrast to a single proline, the additional double proline creates a more rigid peptide backbone, and therefore overrides the advantageous disordering effects of a single proline on dephosphorylation. Furthermore, proline also differs from other amino acids in that it has a significantly higher solubility and thus can dramatically increase the solubility of the peptide sequence. The active site of PP1/PP2A largely excludes solvent waters [86]. Thus, trying to insert the highly soluble Pro-Pro sequence into a solvent poor area may come at a significant energy cost, providing an additional negative impact on dephosphorylation (Figure 2B). In support, switching the second proline for an amino acid with a small non-polar side chain (Gly, Leu, Ala, Val) correlated with favourable dephosphorylation during mitotic exit [22], suggesting that these flexible and less soluble residues help facilitate insertion into the active site and subsequent dephosphorylation (Figure 2C).

More broadly, the secondary structure of the phosphorylation site has a well-established history of regulating mitosis, especially with regard to proline-directed phosphorylation. Both phosphorylated serine and threonine residues adjacent to proline undergo interconversion between *cis* and *trans* forms, albeit at a slow rate [99]. However, *cis-trans* isomerases, such as Pin1, bind to proline-directed phosphosites and catalyse *cis-trans* isomerisation [100,101]. PP2A has been linked to preferentially dephosphorylating the *trans* state of Cdc25C and Tau [98]. This suggests that Pin1 plays a key role in performing isomerization of critical phosphorylation sites into the *trans* state to allow dephosphorylation during mitotic exit. However, Pin1 also has been shown to play a protective role by promoting the *cis* conformation. Specifically, a recent study showed that Pin1 isomerisation of *trans*-separase into the *cis* form during the metaphase-anaphase transition renders it resistant to inhibition by securin, which ensures correct chromatid separation [97]. Notably, cells depleted of Pin1 arrest in mitosis [99], highlighting the importance that isomerisation and therefore the structure of phosphosites plays in regulating dephosphorylation during mitotic exit.

Conclusions

In summary, the temporal ordering of mitotic exit events is controlled by multiple mechanisms that ensure the order of dephosphorylation occurs correctly. At the level of individual phosphosites, this specificity is in part likely dependent on the physico-chemical properties of both the phosphorylated residue, its neighbouring amino acids and how these interact

with the active site of phosphatases. While great advancements in our understanding of which phosphatases are involved in regulating mitotic exit have occurred in recent times, the amount of information is still significantly dwarfed by research on kinases. To date, our best understanding of the phosphatases involved in regulating mitotic exit in higher eukaryotes is limited broadly to the PP1 and PP2A superfamily of phosphatases. However, given that these enzymes can form 70–200 different multimeric complexes, identifying the exact sub-complexes involved in dephosphorylating specific phosphosites is key to fully understanding how phosphatases regulate mitotic exit.

The specific phosphosite information contained within large quantitative phosphoproteomic datasets could be used to aid in the identification of explicit phosphatase-phosphosite pairs. When combined with knowledge of the upstream kinase, this information will provide a more complete understanding of how disruption of one of these factors results in downstream phenotypes. For example, an Aurora B substrate that is rapidly dephosphorylated will be far more likely to be affected than a phosphosite that is poorly dephosphorylated when exposed to an Aurora B kinase inhibitor. Consequently, the combination of the dephosphorylated and stable sites will determine the exact phenotype. However, if the specific phosphatase complex responsible is down regulated or mutated, then the effectiveness of the kinase inhibitor will also be reduced, as the phosphosite will remain phosphorylated. Similarly, mutations near the phosphosite, which alter its affinity (increase or decrease) for a phosphatase, have the potential to affect response to a targeted chemotherapy. These situations, albeit hypothetical, provide a compelling reason for the urgent need to study phosphatases in much greater detail, as they could provide the fundamental information necessary for designing accurate biomarkers of response and potentially identify novel pathways for targeted therapies.

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