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PP2A as a master regulator of the cell cycle

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Abstract

Protein phosphatase 2A (PP2A) plays a critical multi-faceted role in the regulation of the cell cycle. It is known to dephosphorylate over 300 substrates involved in the cell cycle, regulating almost all major pathways and cell cycle checkpoints. PP2A is involved in such diverse processes by the formation of structurally distinct families of holoenzymes, which are regulated spatially and temporally by specific regulators. Here, we review the involvement of PP2A in the regulation of three cell signaling pathways: wnt, mTOR and MAP kinase, as well as the G1→S transition, DNA synthesis and mitotic initiation. These processes are all crucial for proper cell survival and proliferation and are often deregulated in cancer and other diseases.

Keywords

Cancer; cell cycle; cell division; mitosis; phosphatase; PP2A

Introduction

Numerous proteins are involved in regulating the complex processes in cell division, and kinases and phosphatases are the primary regulators. Several kinases and phosphatases are well understood and reviewed in detail (Belle *et al.*, 1990; Bononi *et al.*, 2011; Fisher *et al.*, 2012; Holt, 2012; Hunter, 1995; Mochida & Hunt, 2012). It is clear from early work that the initial focus on cell cycle regulation was kinases, and phosphatases were thought of merely as housekeeping enzymes. More recently, phosphatases are increasingly appreciated for their tight regulation and specific action on key players in the cell cycle (Janssens & Goris, 2001; Virshup & Shenolikar, 2009). One of the most versatile and important phosphatases involved in cell division is protein phosphatase 2A (PP2A). PP2A regulates every stage of the cell cycle in several critical pathways and, not surprisingly, has been widely implicated in tumor suppression (Eichhorn *et al.*, 2009). As such, PP2A is being actively investigated as a therapeutic target (Sangodkar *et al.*, 2015). This review is an attempt to aggregate the numerous substrates dephosphorylated by PP2A and discuss its regulatory activity in major pathways at each stage of the cell cycle.

Protein phosphatase 2A: a complex and diverse family of phosphatases

Background

Eukaryotic phosphatases can be divided into three super families: the serine/threonine phosphatases (PSPs), the tyrosine phosphatases (PTPs) and the dual specificity phosphatases (DSPs) [reviewed in (Shi, 2009; Hunter, 1995; Virshup & Shenolikar, 2009)]. There are around 100 PTPs, approximately equivalent to the number of tyrosine kinases in the genome. Over 400 serine/threonine kinases are expressed in the human proteome (Manning *et al.*, 2002), exceeding that of PSPs by more than 10 fold. Serine/threonine phosphorylation constitutes more than 98% of total protein phosphorylation inside mammalian cells; however, the number of genes encoding PSPs (7) is very small. This controversy is reconciled by the fact that some of the PSPs form a large number of diverse oligomeric complexes. In particular, PP2A forms ~100 heterotrimeric holoenzymes and protein phosphatase 1 (PP1) forms ~400 heterodimeric holoenzymes. Some kinases also form oligomeric complexes, such as cell cycle dependent kinases (CDKs) and mTOR, on a scale much smaller than PP2A and PP1 though, underlying that complex and tight control of both kinases and phosphatases are important for cellular processes.

The PSPs are further divided into three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and aspartate-based phosphatases (Figure 1). The PPP family is the largest family of phosphatases, and many PPPs are involved in cell cycle regulation, including PP2A (Hunter, 1995; Shi, 2009; Virshup & Shenolikar, 2009). The PPP family phosphatases have a structurally conserved active site configuration. Two catalytic metal ions are coordinated by six conserved residues [two aspartate (D), one asparagine (N) and three histidine (H) residues], and a catalytic water molecule. Phosphate binding is coordinated by one conserved histidine and two arginine (R) residues. The dephosphorylation reaction proceeds via an S_N2 mechanism with the activated water serving as a nucleophile to attack the phosphate group attached to Ser or Thr residues (Shi, 2009). PP2A is one of the most complex members in the PPP family, regulating diverse physiological and cellular processes such as neuronal stabilization, cardiac muscle function and the cell cycle. As such, it is implicated in many human diseases such as Alzheimer's disease, cardiac disease and cancer (Eichhorn *et al.*, 2009; Heijman *et al.*, 2013; Kotlo *et al.*, 2012; Martin *et al.*, 2013). PP2A affects such variety of processes due to the formation of diverse heterotrimeric holoenzymes.

Regulation and activation of PP2A

Each PP2A holoenzyme is formed by a combination of three subunits: a common catalytic (C or PP2Ac) subunit containing the active site, a regulatory (B) subunit which confers substrate specificity and a common scaffolding (A) subunit that holds B and C together (Xu *et al.*, 2006). There are two isoforms, α and β , for both A and C, and they share high sequence homology. The α isoform for each is expressed at a much higher level and is the predominant isoform studied in PP2A research. In addition to A and B subunits, cellular PP2Ac is also found associated with $\alpha4$ protein and TOR Signaling Pathway Regulator-like (TIPRL) (Nakashima *et al.*, 2013). Extensive efforts on understanding the structural and biochemical basis of PP2A regulation illuminated a linear pathway for the biogenesis of

PP2A holoenzymes (Figure 2). The exact function of $\alpha 4$ on PP2Ac has been difficult to unravel. Our recent structural evidence suggests it preferentially binds to the partially folded PP2Ac and stabilizes it for stable latency (Jiang *et al.*, 2013). $\alpha 4$ stabilizes PP2Ac in part by protecting it from ubiquitination by Midline 1 (MID1) and preventing its subsequent degradation (Liu *et al.*, 2001; Short *et al.*, 2002). This provides a pool of latent PP2Ac for the biogenesis of diverse heterotrimeric holoenzymes while simultaneously preventing the unregulated phosphatase activity of free PP2Ac and protecting cells from nontargeted dephosphorylation (Jiang *et al.*, 2013).

Consequently, PP2A must be activated before being assembled into active holoenzymes. The phosphotyrosyl phosphatase activator (PTPA), now known as PP2A-specific phosphatase activator, plays a critical role in PP2A activation (Guo *et al.*, 2014). PTPA stabilizes an active conformation of the active site and facilitates the loading of catalytic metal ions (Guo *et al.*, 2014). PP2A together with PTPA forms a combined ATP-binding pocket, which orients the γ -phosphate of ATP to directly chelate catalytic metal ions. Following activation, the phosphatase active site catalyzes ATP hydrolysis. This is crucial for efficient loading of authentic catalytic metal ions and acquisition of pSer/Thr-specific phosphatase activity (Guo *et al.*, 2014). Evidence suggests there is a Zn^{2+} ion in the active site and that ATP is required to load a Mg^{2+} ion into the second position to activate PP2A (Guo *et al.*, 2014).

PP2Ac also undergoes post-translational modification on its unstructured carboxy-terminal tail (Janssens *et al.*, 2008; Lee & Pallas, 2007); phosphorylation on T304 and Y307 and carboxymethylation on L309 (Low *et al.*, 2014). The latter is reversibly controlled by PP2A-specific methyltransferase known as leucine carboxy methyltransferase (LCMT-1), and by PP2A-specific methylesterase 1 (PME-1) (Stanevich *et al.*, 2011; Xing *et al.*, 2008). PP2A methylation is essential for cellular function, and cells will undergo apoptosis in the absence of LCMT-1 (Lee & Pallas, 2007). Reduction in LCMT-1 expression or over-expression of PME-1 can promote transformation through Akt or S6K pathways (Jackson & Pallas, 2012). Methylation is crucial for the formation of stable heterotrimeric B/PR55 family holoenzymes inside cells (Longin *et al.*, 2007), but it is not required for *in vitro* assembly nor is the carboxymethylated PP2Ac tail visible in the PP2A-B α structure (Xu *et al.*, 2008). Carboxymethylation is also not required for *in vitro* assembly of PP2A-B' holoenzymes, but B' holoenzyme structures show the carboxymethylated tail is situated in an area between the A-B interface with several negatively charged residues, suggesting a possible role of methylation in charge neutralization (Cho & Xu, 2007; Xu *et al.*, 2006). Although PP2Ac carboxymethylation is not strictly required for holoenzyme assembly *in vitro*, it is clearly required for proper *in vivo* holoenzyme assembly (Lee & Pallas, 2007; Mumby, 2001). PP2Ac methylation also fluctuates during the cell cycle, indicating that regulation of PP2Ac methylation and holoenzyme assembly is required for cell cycle regulation (Janssens *et al.*, 2008; Yu *et al.*, 2001).

Structural diversity of holoenzymes

Protein phosphatase 2A (PP2A) can act on a wide range of substrates via its diverse holoenzymes, each containing a distinct B subunit from four families: the B (PR55), B' (PR56), B'' (PR72) and B''' (Striatin) (Shi, 2009). Currently, the identified regulatory

subunits are encoded by 15 genes which can be alternatively spliced to yield 26 different B subunits (Eichhorn *et al.*, 2009). A summary of subunit nomenclature can be found in Table 1. These subunits share sequence homology within each family, but have little-to-no sequence homology between the families (Eichhorn *et al.*, 2009). No specific “recognition motif” has been identified for PP2A substrates, and the recognition is likely due to structural elements inherent to each subunit. The structure of the core dimer of PP2A revealed important insights on how holoenzyme assembly and activity are regulated (Xing *et al.*, 2006). The core of PP2Ac contains two central β -sheets flanked by α -helices, with the loops connecting to the β -sheets forming the active site, and the active site loops harbor six conserved residues that chelate catalytic metal ions. The active site loops are highly dynamic (Guo *et al.*, 2014; Jiang *et al.*, 2013). As such, all holoenzyme (and core enzyme) structures solved to date required potent inhibitors such as microcystin LR (MCLR) or okadaic acid (OA) to stabilize the active site for crystallization (Wlodarchak *et al.*, 2013; Xing *et al.*, 2006; Xu *et al.*, 2006,2008,2009).

The A-subunit consists of 15 Huntington-elongation-A-subunit-TOR (HEAT) repeats arranged in a horseshoe shape. PP2Ac binds to the ridge region of repeats 10–15 and the regulatory subunits bind to the ridge of the N-terminal repeats. The A-subunit can undergo a large degree of conformational changes, explaining how so many structurally diverse B subunits can form active holoenzymes with the same A–C dimer (Wlodarchak *et al.*, 2013). The B' γ 1 holoenzyme was the first holoenzyme structure solved (Xu *et al.*, 2006). Similar to the A-subunit, the B' γ 1 subunit is also a HEAT repeat protein. The structure of the B α holoenzyme demonstrated a much wider conformation for the A-subunit than the B' holoenzyme, with little interaction between the B and C subunits (Xu *et al.*, 2008). The B α subunit is a 7-bladed β -propeller with a hairpin that extends to interact with the side face of the N-terminal HEAT repeats of the A-subunit (Xu *et al.*, 2008). Recently, the high-resolution structure of a B'' holoenzyme associated with PR70 and two structures of B'' family subunits in isolation were finally solved (Dovega *et al.*, 2014; Wlodarchak *et al.*, 2013). These structures show that the B'' subunits are distinct from other families and consist of a multi-domain arrangement with two prominent calcium binding EF hands and a hydrophobic interacting motif. One of the EF hands directly contacts the top ridge of the scaffold subunit and is important for A–B'' binding (Dovega *et al.*, 2014; Wlodarchak *et al.*, 2013). The N-terminal hydrophobic motif binds to the N-terminal side surface of the A-subunit while the C-terminal domain interacts with PP2Ac. These tripartite contacts between AC and PR70 force the A-subunit into a tight conformation, and this is required for enhanced substrate-specific dephosphorylation (Wlodarchak *et al.*, 2013). These observations suggest that precise orientation of substrates is dependent on subtle structural features and compact conformation of the holoenzymes derived from large conformational changes in the A-subunit. Future structural and functional studies are required to illuminate these mechanisms in more detail.

Given the diversity of holoenzyme structures, it is no surprise that PP2A has been suggested or confirmed to dephosphorylate over 300 substrates (Table 2). Most of these substrates are involved in cell cycle regulation, and although some of PP2A-mediated dephosphorylation cause positive regulation of proliferation pathways, the majority of PP2A-mediated dephosphorylation events play a negative regulatory role. PP2A is implicated in a wide array

of human diseases due to its prominent function in the cell cycle and many other essential cellular processes.

The cell cycle initiation: signaling pathways

The initiation of the cell cycle is controlled by many diverse and complex signaling pathways. There is a large and incredibly detailed body of information on each of these signaling pathways elsewhere. Presented here is a brief review on three critical signaling pathways, Wnt, mTOR and MAPK, with a focus on the role of PP2A in their regulation.

Wnt signaling pathway

The Wnt pathway is involved in the regulation of cell proliferation and polarity as well as embryonic development. It facilitates the initiation of the cell cycle by activating the transcription of critical promoters of cell division such as cyclin D1 and c-Myc (He *et al.*, 1998; Rimerman *et al.*, 2000). In the absence of Wnt signaling (Figure 3A), the protein β -catenin is degraded by the action of a complex composed of axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 β (GSK3 β) and casein kinase 1 (CK1) [reviewed in Clevers & Nusse (2012) and MacDonald *et al.* (2009)]. GSK3 β and CK1 phosphorylate β -catenin, targeting it for ubiquitination and proteasomal degradation (Amit *et al.*, 2002; van Noort *et al.*, 2002). APC and axin have unique domains that bind to CK1 and GSK3 β to serve as scaffolds to increase β -catenin phosphorylation, and these scaffolds are often found mutated in cancers (Dajani *et al.*, 2003; Kinzler *et al.*, 1991; Nishisho *et al.*, 1991). Wnt signaling (Figure 3B) is activated when extracellular Wnt binds to the receptor frizzled and co-receptor LRP 5/6. An intracellular complex is then formed with the receptors disheveled, axin, CK1 and GSK3 β , which then prevents the phosphorylation and subsequent degradation of β -catenin (Julius *et al.*, 2000). β -Catenin can then accumulate in the nucleus and bind to TCF family transcription factors and activate Wnt responsive genes (Behrens *et al.*, 1996). These include critical promoters of cell division such as cyclin D1 and c-Myc (He *et al.*, 1998; Rimerman *et al.*, 2000).

β -Catenin is the central substrate in Wnt signaling, and its regulation is highly dependent on phosphorylation and dephosphorylation. The phosphorylation events are sequential, with CK1 phosphorylating S45 followed by GSK3 β phosphorylating T41, S37 and S33 (Amit *et al.*, 2002; van Noort *et al.*, 2002). Phosphorylation at S37 and S33 allows the ubiquitin ligase β -transducin repeat containing protein (β -TRCP) to bind β -catenin and target it for degradation (Latres *et al.*, 1999). In addition to phosphorylating β -catenin, CK1 and GSK3 β can phosphorylate APC and axin which increases the affinity of β -catenin for these scaffolds (Ferrarese *et al.*, 2007; Ha *et al.*, 2004; Jho *et al.*, 1999). These phosphorylation events are disrupted when the complex is perturbed by Wnt signaling. β -catenin, APC and axin can be dephosphorylated by phosphatases such as PP2A and PP1, and this event can also increase β -catenin levels. PP1 increases β -catenin levels by dephosphorylating axin which reduces its affinity for GSK3 β (Luo *et al.*, 2007). Unlike PP1, PP2A has a dual and opposing role in β -catenin regulation (Figure 3). The PP2A-B α holoenzyme has been shown to directly interact with and dephosphorylate β -catenin to enhance Wnt signaling (Zhang *et al.*, 2009). In addition to dephosphorylating the residues relevant to destruction, this holoenzyme can also

dephosphorylate residues S552 and S675, the functionality of which has yet to be elucidated (Zhang *et al.*, 2009). The B55 α holoenzyme can also directly bind axin, likely through a different domain than the one that PP2Ac can bind (Zhang *et al.*, 2009). In contrast, the PP2A B' α holoenzyme has been implicated in negative regulation of Wnt signaling (Figure 3). B' α can bind to the destruction complex through APC. Overexpression of B' α results in decreased β -catenin levels and the amino terminus of β -catenin being required for this effect (Seeling *et al.*, 1999). In addition to β -catenin regulation, PP2A negatively regulates Wnt signaling through GSK3 β both directly and indirectly (Figure 3). GSK3 β is inhibited by phosphorylation on S9 by AKT (Leung-Hagesteijn *et al.*, 2001). DNAJB6 with Heat Shock Cognate 40 (HSC40) can recruit PP2A to GSK3 β where it can directly dephosphorylate S9 and activate GSK3 β (Mitra *et al.*, 2012), which targets more β -catenin for destruction (Kumar *et al.*, 2012). PP2A also inhibits Protein Kinase B (AKT or PKB) (Kumar *et al.*, 2012), which indirectly activates GSK3 β by downregulating phosphorylation on S9. This pathway also provides an important intersection with the mTOR pathway, another critical cell cycle initiating pathway.

Mechanistic target of rapamycin (mTOR)

The mTOR pathway is involved in many diverse cellular processes. It is stimulated by amino acids, cellular metabolism, and growth factors, and results in increased growth, metabolism and biomolecule synthesis (Laplante & Sabatini, 2012). These are crucial for accumulating enough cellular components required for cell division. Due to the multifaceted role of mTOR in cell regulation, it is an intensely studied pathway with major implications in cancer, heart disease and even some neurological diseases such as autism (Chen *et al.*, 2014; Fruman & Rommel, 2014; Laplante & Sabatini, 2012; Sciarretta *et al.*, 2014;). Rapamycin was known to have toxic effects on yeast, and the responsible genes (DDR1&2/TOR1&2) were discovered in 1993, with the protein being discovered 1 year later (Brown *et al.*, 1994; Cafferkey *et al.*, 1993; Kunz *et al.*, 1993). Two complexes are formed with the mTOR catalytic protein: mTORC1 and mTORC2 (Shimobayashi & Hall, 2014). Both complexes contain some shared as well as some unique components. The shared components are the tti1 and tel2 scaffolds, deptor and mLST8 (Shimobayashi & Hall, 2014). mTORC1 contains the unique proteins raptor and pras40, whereas mTORC2 contains rictor, mSin1 and protor1/2 (Laplante & Sabatini, 2012; Shimobayashi & Hall, 2014). Deptor is an inhibitor of both mTOR complexes and suppresses the function of S6 kinase 1 (S6K1), AKT and Serum and Glucocorticoid regulated kinase 1 (SGK1) (Peterson *et al.*, 2009). Deptor is highly overexpressed in some multiple myelomas, and this overexpression can induce AKT function due to loss of feedback inhibition of phosphoinositide-3 kinase (PI3K) from mTORC1 (Peterson *et al.*, 2009). Raptor and rictor help regulate substrate specificity to mTORC1 and mTORC2, respectively. Raptor binds with mTOR in the mTORC1 complex and is necessary for binding and phosphorylation of S6K1 and 4E-BP1, which in turn induce protein synthesis and proliferation (Kim *et al.*, 2002; Nojima *et al.*, 2003). G β L (also known as mLST8) is found in both mTORC2 and mTORC1 and is essential in stabilizing the interaction of mTOR with raptor (Kim *et al.*, 2003; Laplante & Sabatini, 2012).

Both complexes are regulated by highly diverse processes. Wnt signaling can activate both mTOR complexes, as can stimulation by insulin (Inoki *et al.*, 2006; Shimobayashi & Hall,

2014). Wnt can stimulate mTORC2 directly through the GTPase RAC1 and stimulates mTORC1 indirectly by inactivating glycogen synthase kinase 3 β , which is necessary to activate an mTORC1 inhibitor, tuberous sclerosis complex 2 (TSC2) (Inoki *et al.*, 2006; Shimobayashi & Hall, 2014). Insulin, long known to stimulate protein synthesis, activates mTOR through a central molecule, phosphatidylinositol-3,4,5-triphosphate, produced by PI3K (Hsu *et al.*, 2011; Shimobayashi & Hall, 2014). This molecule can directly stimulate mTORC2 and can indirectly stimulate mTORC1 by activation of AKT which inhibits TSC2 (Klippel *et al.*, 1997; Shimobayashi & Hall, 2014). Free amino acids activate mTORC1 via binding to RAS-related GTP binding protein (RAG) heterodimers, causing a global conformational change (Sancak *et al.*, 2008). The RAG complex associated with amino acids can recruit mTORC1 to the lysosome where it is activated by binding to RAS homolog enriched in brain (RHEB) (Sancak *et al.*, 2008; Shimobayashi & Hall, 2014).

Although the mTOR regulation pathways are well established, the downstream substrates of mTOR are not very well characterized. Mass spectrometric studies have identified 93 potential substrates in human embryonic kidney cells and 174 potential substrates in mouse embryonic fibroblasts (Hsu *et al.*, 2011). Very few of these substrates have been studied in detail. The best known substrates of mTOR are the ribosomal S6 kinase (S6K) and eIF4e binding protein 1 (4E-BP) (Shimobayashi & Hall, 2014). Many proteins that are involved in growth and proliferation are encoded by mRNAs that have secondary structures in their 5'-UTR (untranslated region) that inhibit scanning by the 40S ribosomal subunit [reviewed in Ma & Blenis (2009)]. Phosphorylation of 4E-BP by mTORC1 can inhibit its binding to eIF4E, which is necessary to recruit the pre-initiation complex (Burnett *et al.*, 1998; Ma & Blenis, 2009). An important component of the pre-initiation complex is eIF4B, which upon phosphorylation recruits eIF4A, a family of RNA helicases that facilitate efficient unwinding of secondary mRNA structures. eIF4B phosphorylation is mediated by S6K after phosphorylation and activation by mTORC1 (Burnett *et al.*, 1998; Ma & Blenis, 2009). These mTOR substrates both work in concert to translate these structured mRNAs to enhance growth and proliferation. mTOR regulates autophagy by the phosphorylation of Unc 51-like kinase 1 (ULK1). Autophagy is promoted by ULK1 when activated by phosphorylation on S317 and S777 by AMP activated protein kinase (AMPK) (Kim *et al.*, 2011). mTOR phosphorylates ULK1 on S757, which prevents AMPK phosphorylation and subsequent activation (Kim *et al.*, 2011). Recently, LIPIN1, a protein that helps promote lipid biosynthesis, was identified as a potential mTOR substrate, but more work is needed to further characterize this substrate (Yuan *et al.*, 2012).

There is evidence that PP2A can associate and dephosphorylate S6K, and the same report indicated that mTOR can inactivate PP2A, providing two modes of S6K activation (Peterson *et al.*, 1999) (Figure 4). PP2A has also been shown to affect mTOR activity by regulating AKT. AKT, which inhibits TSC2, requires phosphorylation on T308 and S473 for activation, and the PP2A B' α holoenzyme has been shown to dephosphorylate AKT on T308, thereby inactivating it (Kuo *et al.*, 2008) (Figure 4). Upstream of AKT, driven by insulin signaling, is insulin receptor substrate 1 (IRS1) which is necessary to transduce insulin receptor signaling to PI3K (Carlson *et al.*, 2004). PP2A can dephosphorylate IRS1, leading to its stabilization and mTOR can inhibit PP2A activity toward IRS1 potentially directly phosphorylating IRS1

at S307, leading to IRS1 degradation (Carlson *et al.*, 2004; Hartley & Cooper, 2002) (Figure 4).

Multiple reports have indicated that mTOR can negatively regulate PP2A activity, and most of this negative regulation supports mTOR activation through insulin signaling and PI3K. In contrast, PP2A can negatively regulate mTOR when amino acids are not present. MAP4K3 can signal to activate mTOR when amino acids are present, and autophosphorylation on S170 is necessary for this activation (Yan *et al.*, 2010). When amino acids are withdrawn, PP2A dephosphorylates S170 and prevents mTOR activation by this pathway (Yan *et al.*, 2010) (Figure 4).

The role of PP2A in mTOR activation is further regulated by its regulatory proteins. TIPRL can overcome amino acid withdrawal and stimulate mTOR activation by inhibiting PP2A phosphatase activity. However, the yeast homolog, TIP41, has a negative effect on mTOR activation by binding to TAP42 (Nakashima *et al.*, 2013). In contrast to observations in yeast, the association between PP2Ac and $\alpha 4$, the mammalian homolog of TAP42, is not dependent on mTOR, indicating that the functions of TIPRL and $\alpha 4$ in mTOR signaling are not conserved and remain to be deciphered (Yoo *et al.*, 2008).

The mTOR pathway is a critical pathway to initiate cell growth, and the regulation of this pathway is exceedingly complex, involving many feedback loops and antagonistic partnerships, especially with PP2A. mTOR has been intensely investigated and is frequently targeted for potential treatment of diseases such as cancer. Nevertheless, there are still important gaps in our understanding of mTOR substrates, regulation and crosstalk with other signaling pathways. Further study will deepen our understanding of growth signaling and possibly lead to significant drug development.

Mitogen activated protein kinase signaling pathway

Mitogen-activated protein (MAP) kinase pathways help regulate many cellular functions such as proliferation, differentiation and apoptosis. There are four families of MAP kinases: ERK1/2, ERK5, JNK and p38 [reviewed in Hommes *et al.* (2003), Imajo *et al.* (2006), McCubrey *et al.* (2007), Meloche & Pouyssegur (2007)]. When activated, MAP kinases phosphorylate downstream substrates to induce cellular responses. MAPKs are activated by upstream kinases (MAPK kinases), and those MAPKKs are activated by further upstream kinases, MAPKK kinases (MAPKKKs) (Imajo *et al.*, 2006). These kinases are activated by cellular growth signals, cytokines or stress signals. ERK5, JNK and p38 generally have pro-apoptotic functions and are activated by stresses, whereas ERK1/2 promotes proliferation and transformation (Imajo *et al.*, 2006; Meloche & Pouyssegur, 2007; Wu, 2007). The ERK1/2 pathway was the first MAPK pathway discovered and is the best studied. Growth factors such as epidermal growth factor (EGF) or fibroblast growth factor (FGF) bind to their respective receptors and recruit a complex of SRC homology-2-containing protein (SHC), growth factor receptor bound protein 2 (GRB2) and son of sevenless (SOS) (Egan *et al.*, 1993) (Figure 5). This complex changes Ras conformation, disrupting GDP interaction and promoting GTP association which activates Ras and recruits Raf (MAPKKK) to the membrane bound complex (Freedman *et al.*, 2006; Milburn *et al.*, 1990), where Raf is activated by dimerization and phosphorylation (Rajakulendran *et al.*, 2009). Raf then

phosphorylates and activates MEK1, which subsequently phosphorylates and activates ERK1/2 (Crews *et al.*, 1992; Wu *et al.*, 1996) (Figure 5). ERK1/2 phosphorylates transcription factors Jun and Fos which can then translocate to the nucleus and bind DNA to initiate transcription of genes involved in cell cycle regulation such as AP-1, which in turn can promote cyclin D1 expression (Monje *et al.*, 2005; Weber *et al.*, 1997). ERK1/2 can also phosphorylate and stabilize c-Myc, which can then enhance its transcriptional activity toward cell cycle promoting genes such as cyclin D1 and CDC25A (Mathiasen *et al.*, 2012; Meloche & Pouyssegur, 2007).

The ERK1/2 MAPK pathway and its downstream substrates are also regulated by the action of phosphatases. There are at least 11 MAPK phosphatases (MKPs), which are split into three families based on cellular localization. There is significant cross-activity between the MKPs and all four of the MAPK pathways, and many of the MKPs, such as MKP-1 and MKP-3, have an overall transforming effect and are implicated in many cancers [reviewed in Wu (2007)]. MKP-1 has been the best studied of the MKPs, and is implicated in a variety of cancers. One of the mechanisms implicated in maintaining cell survival is its ability to prevent stress-induced apoptosis by preferentially dephosphorylating p38 and JNK, inactivating two critical stress-induced apoptotic pathways in the cell (Franklin & Kraft, 1997; Franklin *et al.*, 1998).

Protein phosphatase 2A (PP2A) appears to have a role primarily in negative regulation of the ERK MAPK pathway (Figure 5). SHC is an important member of the complex that binds growth receptors and activates Ras (Egan *et al.*, 1993). PP2A can bind to the phosphotyrosine binding domain of SHC and negatively regulate Ras activation (Ugi *et al.*, 2002). After growth factor stimulation, T317 phosphorylation of SHC can dissociate PP2A and allow downstream activation (Ugi *et al.*, 2002). It is currently unknown whether PP2A actively dephosphorylates SHC or which regulatory subunit is responsible for PP2A's inhibitory effect (Ugi *et al.*, 2002). PP2A can also directly inactivate ERK by dephosphorylation (Letourneux *et al.*, 2006) (Figure 5). This is mediated by the B' β and B' γ subunits, which can also be phosphorylated by ERK if IEX-1 is expressed, thus reversing PP2A mediated inactivation (Letourneux *et al.*, 2006). Sprouty2 is an inhibitor of FGF stimulated ERK activation. Sprouty2 is normally phosphorylated and cannot bind Grb2, and phosphorylation on T55 allows c-Cbl to bind and target sprouty2 for degradation by the proteasome (Lao *et al.*, 2007). Upon FGF stimulation, sprouty2 is dephosphorylated by PP2A, which exposes the Grb2 binding motif on the C-terminus (Lao *et al.*, 2007) (Figure 5). When bound to Grb2, ras is unable to be recruited to the complex and be activated, thus downregulating ERK activation by FGF (Egan *et al.*, 1993). PP2A binds to sprouty2 between residues 50–60, competing with c-Cbl binding and thus activating and protecting sprouty2 (Lao *et al.*, 2007).

PP2A can also positively regulate ERK MAPK signaling. EGF receptors are targeted for ubiquitination and degradation by c-Cbl, which requires phosphorylation on various residues (McCubrey *et al.*, 2007). This interaction is disrupted upon recruitment of SRC homology 2 domain containing inositol polyphosphate phosphatase, SHIP2 (Zwaenepoel *et al.*, 2010). PR130, a PP2A regulatory subunit from the B'' family, forms a holoenzyme which can form a complex with SHIP2 and is required for SHIP2-mediated stabilization of EGFR

(Zwaenepoel *et al.*, 2010). Mapping studies indicate that the catalytic domain of SHIP2 interacts with the EF hands of PR130, and mutation in this region disrupts the stabilizing effect of PR130 holoenzyme on EGFR (Zwaenepoel *et al.*, 2010). It is currently unknown whether catalytic activity is required for this effect and whether PP2A-PR130 dephosphorylates EGFR, SHIP2 or other associated targets. Downstream of growth factor receptor, the kinase suppressor of ras (KSR1) is a critical positive regulator of ras signaling (Ory *et al.*, 2003). It is a necessary scaffold to transduce the activation signal from ras-1 to MEK to ERK. PP2A-B α holoenzyme is associated with the KSR1 complex and is required for MEK activation (Ory *et al.*, 2003). When phosphorylated, S392 of KSR1 associates with 14-3-3 protein and remains cytoplasmic. PP2A-B α directly dephosphorylates KSR1 at S392 which is then freed from 14-3-3 and can translocate to the membrane, an event required for MEK activation (Ory *et al.*, 2003) (Figure 5). Similarly, PP2A and PP1 have been shown to positively regulate Raf-1 activity by dephosphorylating S259, allowing 14-3-3 release from Raf-1 and membrane translocation (Jaumot & Hancock, 2001) (Figure 5). PP2A-B β and -B δ can also positively regulate MAPK signaling in neuronal PC12 cells through action on TrkA. PP2A enhances autophosphorylation of TrkA likely by dephosphorylating an inhibitory Ser/Thr, allowing Ras activation by TrkA and sustained MAPK signaling (Van Kanegan & Strack, 2009).

Taken together, the role of PP2A in regulation of MAPK pathway is complex and other as-yet-unidentified regulatory proteins may be involved. Signaling scaffolding proteins, such as KSR1, are crucial for coordinating spatiotemporal control of the function of kinases, phosphatases and other signaling molecules. The role of PP2A in both positive and negative regulation of MAPK are likely crucial for fine-tuning and precise control of this pathway. There is also crosstalk with many of these pathways as well as other cell cycle promoting pathways, highlighting the importance of phosphatases in regulating the initiation of the cell cycle and the complexity by which they do so.

Cell cycle progression: Rb and the G1-S transition

In G1 phase, cell cycle initiation pathways, such as those mentioned above, initiate growth and transcription of factors, such as cyclin D1, that control cell cycle progression. Before the cell can transition from G1 to synthesis (S) phase, it must pass through a critical cell checkpoint to ensure that the cell is ready for DNA synthesis. The master regulator of this checkpoint, and first discovered tumor suppressor protein, is the retinoblastoma tumor suppressor protein (Rb).

Rb phosphorylation

Retinoblastoma tumor suppressor protein (Rb) is an approximately 105 kDa protein consisting of three functional domains: an N-terminal structured region, a two-part central pocket region and a C-terminal unstructured region (Harbour & Dean, 2000). There are two other proteins structurally and functionally related to Rb: p107 and p130. Together, these proteins make up the pocket protein family and have all been implicated in diverse cellular processes such as cell cycle progression, apoptosis, senescence, differentiation, and angiogenesis [reviewed in Indovina *et al.* (2013)]. These proteins bind and inactivate E2F

transcription factors, with Rb binding E2F 1–3, and p107/p130 binding E2F4 & 5 (Figure 6) (Indovina *et al.*, 2013; Kolupaeva & Janssens, 2013). E2F 1–3 are transcriptional activators and mostly express cell cycle genes such as cyclins E and A, and CDC25 [reviewed in Harbour & Dean (2000), Indovina *et al.* (2013), Nevins (2001)]. E2F4 & 5 are transcriptional repressors and are involved in maintaining genomic stability and redundant functions with Rb [reviewed in Dominguez-Brauer *et al.* (2010) and Plesca *et al.* (2007)]. The pocket proteins bind to E2F transcription factors along with their dimerization partners (DPs), preventing their translocation to the nucleus and transcriptional activation (Rubin, 2013). The pocket proteins are phosphorylated by cyclin/CDK holoenzymes on numerous residues, weakening the interaction between them and the E2Fs, causing dissociation and E2F transcriptional activation [reviewed in Kolupaeva & Janssens (2013) and Rubin (2013)] (Figure 6). Rb is phosphorylated by cyclin D/CDK4 and cyclin E/CDK2 in G1, and numerous phosphorylation events gradually lead to the release of E2F transcription factors (Brown *et al.*, 1999). One possible mechanism for this gradual release is due to the association of E2F with multiple domains of Rb (Rubin *et al.*, 2005). The pocket domain alone is not sufficient for E2F dimerization, as the C-terminal region of Rb adopts a stable conformation upon association with E2F1-DP and increases the binding of the complex more than 36 fold (Rubin *et al.*, 2005). Loss of this interaction pre-disposes Rb-E2F to dissociate, and this region is phosphorylated by cyclin D/CDK4/6 early in G1, thus providing a model for how sequential phosphorylation events dissociate Rb-E2F (Rubin *et al.*, 2005). Rb levels do not change throughout the cell cycle, indicating that phosphorylation events need to be reversed to reset Rb after cell division (Kolupaeva & Janssens, 2013).

Phosphatases in Rb regulation

The specific roles of kinases in Rb phosphorylation have been well established; however, the role of phosphatases in Rb regulation continues to be discovered. Protein phosphatase 1 and PP2A are the primary phosphatases that regulate Rb (and p107/130) function (Kolupaeva & Janssens, 2013). PP1 is responsible for complete dephosphorylation of Rb after mitosis, whereas PP2A functions throughout the cell cycle, dephosphorylating Rb and p107/130 in response to various stimuli [reviewed in Kolupaeva & Janssens (2013) and Kurimchak & Grana (2012)].

Protein phosphatase 1 (PP1) and PP2A are both known to dephosphorylate Rb, and PP1 appears to compete for the same CDK docking sites (Alberts *et al.*, 1993; Hirschi *et al.*, 2010). Such competitive interaction has also been suggested between CDKs and PP2A for p107 (Kolupaeva *et al.*, 2013), and when CDKs are elevated they outcompete phosphatases causing an irreversible cell cycle progression signal switch. CDKs compete with phosphatases and switch the signal toward hyperphosphorylation and irreversible progression of the cell cycle (Garriga *et al.*, 2004). In support of the competition hypothesis, specific overexpression of B α induces p107 dephosphorylation (Jayadeva *et al.*, 2010). B α can directly associate with p107 but has little affinity for pRb, therefore, additional holoenzymes may be required to mediate cell cycle arrest by pocket protein activation (Jayadeva *et al.*, 2010).

In contrast to constitutive competitive interactions, extracellular signaling or stress factors can induce PP2A-modulated dephosphorylation of the pocket proteins with no significant changes in CDK activity or PP2A expression (Cicchillitti *et al.*, 2003). Sustained FGF signaling can arrest cell growth in chondrocytes, which is the opposite to the effect in most other cell types (Kolupaeva *et al.*, 2013). FGF signaling leads to B α dephosphorylation, increasing B α affinity for p107, and chondrocytes have a large constitutively expressed B α population (Kolupaeva *et al.*, 2013). Phosphorylation of B α allows increased association of PP2A-B α holoenzymes with p107 and subsequent dephosphorylation and cell cycle arrest (Kolupaeva *et al.*, 2013). The extracellular factor all-*trans*-retinoic acid (ATRA) appears to induce PP2A-specific dephosphorylation of p130 (Purev *et al.*, 2011). Upon ATRA treatment, PP2A can bind to and dephosphorylate p130, protecting it from ubiquitination and degradation (Purev *et al.*, 2011). PP2A can also mediate p130's translocation to the nucleus due to dephosphorylation of S1080 and T1097, exposing the NLS and allowing binding by importins α and β (Purev *et al.*, 2011; Soprano *et al.*, 2006). Under oxidative stress conditions, the PP2A-PR70 holoenzyme can dephosphorylate Rb, and this activity is dependent on Ca²⁺ stimulation (Magenta *et al.*, 2008) (Figure 6). One potential underlying mechanism is that oxidative stress induces an influx of Ca²⁺, which stimulates PR70 holoenzyme formation and results in specific Rb dephosphorylation by PP2A-PR70, although further investigation is needed.

The regulation of pocket proteins by dephosphorylation is complex and results from the interplay between competition with CDKs and specific mitogenic or stress stimuli. There may also be crosstalk between various signaling pathways in this process, as pocket proteins are central effectors through which many pathways funnel. S phase induction by Rb represents a commitment by the cell to DNA synthesis and phosphatases continue to be important in regulating this process.

DNA synthesis and regulation of the origin recognition complex

Once the cell passes the G1-S checkpoint, it is committed to the process of synthesizing DNA. The genome in eukaryotes is far too large for synthesis to proceed in a linear fashion from one end to another, so synthesis proceeds from discrete origins of replication. In yeast, these origins are defined by specific DNA sequences; however, human origins are likely defined by DNA structural features [reviewed in Hyrien *et al.* (2013)]. Excluding DNA recognition, the origin recognition complex functions in a conserved manner and is highly regulated.

ORC assembly and regulation

The origin recognition complex (ORC) is a large protein complex that binds to DNA at the origins of synthesis and recruits all of the proteins required to unzip and polymerize DNA [reviewed in Bell (2002) and Duncker *et al.* (2009)]. There are six ORC proteins (ORC1–6) that bind to DNA at the origins [referred to as autonomously replicating sequences (ARS) in yeast] (Duncker *et al.*, 2009). These proteins all bind and hydrolyze ATP, and ATPase activity is required for their assembly and recruitment of other complex members (Bell & Stillman, 1992). In late G1/early S, cell division control 6 protein (Cdc6) binds to the ORC

proteins and is the critical component for further ORC assembly (Liang *et al.*, 1995). Cdc6 facilitates the loading of Cdt1 and ORC6 to ORC1–5 which then facilitate the loading of the mini-chromosome maintenance proteins (MCM2–7) (Nishitani *et al.*, 2000). Cdc6 also has ATPase activity, and hydrolysis of ATP leads to conformational changes, which increases the binding affinity of the MCM proteins for the complex (Shin *et al.*, 2003). The MCM proteins are helicases, and when properly bound they begin to unwind DNA for replication. The MCM proteins are subsequently phosphorylated by Dbf4/Cdc7 which allows the recruitment of RPA and Cdc45 to the unzipped origin, facilitating the loading of DNA polymerase which then synthesizes new DNA (Sheu & Stillman, 2006; Tanaka & Nasmyth, 1998).

Regulation of Cdc6 by phosphorylation

For error free cell division, DNA must be synthesized once and only once. One of the chief ways the cell regulates this process is by allowing the origins to fire only once. This restriction is accomplished by the tight regulation of Cdc6. In the absence of phosphorylation near its N-terminal destruction motifs, the anaphase-promoting complex/cyclosome (APC/C) targets Cdc6 for ubiquitination and proteasomal degradation (Mailand & Diffley, 2005). In G1, Cdc6 is phosphorylated by cyclin E/CDK2 on S54 and S74, protecting it from degradation and allowing it to be transported into the nucleus and to bind to the ORC (Jallepalli *et al.*, 1997; Mailand & Diffley, 2005) (Figure 7). After origin firing in early S phase, Cdc6 is acetylated by general control non-derepressible 5 (GCN5) and phosphorylated by cyclin A/CDK2 on S106 (Paolinelli *et al.*, 2009; Petersen *et al.*, 1999). These modifications tag Cdc6 for nuclear export where it is degraded in the cytoplasm. Cdc6 degradation can only happen when the protective residues are dephosphorylated, and PP2A-PR70 has been shown to dephosphorylate Cdc6 *in vivo* (Davis *et al.*, 2008). Our recent study showed that Cdc6 is specifically dephosphorylated by PP2A-PR70 holoenzyme and not others (Wlodarchak *et al.*, 2013). PP2A-PR70 binds Cdc6 near the phosphorylated residues, likely due to a charge recognition pattern, and a compact holoenzyme conformation is critical for optimal enzymatic activity (Wlodarchak *et al.*, 2013). The *in vivo* timing and location of PP2A-PR70 dephosphorylation is not fully characterized, but it likely occurs after origin firing to prevent re-assembly and possibly before origin assembly to regulate synthesis (Figure 7).

Mitosis: PP2A as a gatekeeper from mitotic entry to mitotic exit

Inhibition of PP2A is required for mitotic entry

The transition from G2 to M phase is elicited by many factors and pathways, but one of the most critical events is the activation of CDK1, which is concomitant with the inactivation of PP2A-B55 holoenzyme (Mochida *et al.*, 2009). The role of CDK1 was discovered over 40 years ago, but the complex regulatory pathways in which it is involved continue to be studied (Fisher *et al.*, 2012). CDK1 is kept inactive by phosphorylation of S14 and Y15 by Wee1 and Myt1 (Mueller *et al.*, 1995). During the G2→M transition, CDK1 is activated by a group of dual-specificity phosphatases, Cdc25a, b and c (herein collectively referred to as CDC25), which themselves are subject to a complex regulatory network involving several kinases and phosphatases (Lammer *et al.*, 1998) [reviewed in Johnson & Kornbluth (2012)]. Before mitotic entry, CDC25 is phosphorylated on S216 by CaMKII and can also be

phosphorylated by Chk1 to arrest the cell cycle (Hutchins *et al.*, 2003). This allows 14–3–3 protein to associate with CDC25 and prevent its nuclear translocation (Margolis *et al.*, 2006a). Chk1 also phosphorylates PP2A-B56 δ on S37 which subsequently enhances its activity toward pT138 of CDC25, keeping CDC25 inactive (Margolis *et al.*, 2006a). At the end of G2, CDK2-cyclin E phosphorylates CDC25 T138, decreasing the affinity of 14–3–3 to CDC25 (Margolis *et al.*, 2006a). The decreased affinity allows gradual 14–3–3 dissociation, and the free 14–3–3 becomes bound in a phospho-keratin sink (Margolis *et al.*, 2006a). The re-exposed S216 can then be dephosphorylated by PP1, preventing 14–3–3 re-association (Margolis *et al.*, 2003,2006b). The now active CDC25 can dephosphorylate pT14 and pY15 of CDK2, subsequently activating it (Gautier *et al.*, 1991). Once CDK1 is active, it can phosphorylate CDC25 at S214, enhancing the affinity of CDC25 for PP1 and causing activation of additional CDK1, leading to rapid mitotic progression (Margolis *et al.*, 2006b) (Figure 8).

Protein phosphatase 2A (PP2A)-B55 holoenzyme provides additional mechanism for the complex CDK1 regulation. Before mitotic initiation, PP2A-B55 dephosphorylates Wee1 and Greatwall kinase, keeping both inactive (Harvey *et al.*, 2011; Hegarat *et al.*, 2014). It can also dephosphorylate and subsequently inactivate CDC25 at mitotic exit (Forester *et al.*, 2007; Johnson & Kornbluth, 2012). In addition, cyclin A-CDK2 begins to phosphorylate Greatwall kinase at T194 and activate it at the G2→M transition (Hegarat *et al.*, 2014). Greatwall phosphorylates ENSA that subsequently binds to and inhibits PP2A-B55, preventing CDC25 repression (Mochida *et al.*, 2010). As more CDK1 is activated, a positive feedback loop ensures PP2A-B55 inactivation. CDK1 also phosphorylates Greatwall and FCP1, keeping a majority of ENSA phosphorylated and bound to PP2A-B55 (Hegarat *et al.*, 2014). Furthermore, CDK1 inactivates Wee1 and, without the antagonistic effect of PP2A-B55, ensures its activation (Watanabe *et al.*, 2005). This complex network of regulation and positive feedback loops serve to inactivate PP2A while activating CDK1, rapidly driving entry into mitosis.

PP2A in reorganization of cellular structures during mitosis

Protein phosphatase 2A (PP2A) holoenzymes play a critical role in regulating reorganization of cellular structures during mitosis, including nuclear envelope breakdown, rearrangement of intracellular organelles, such as the endoplasmic reticulum and the Golgi apparatus, assembly of mitotic chromosomes, assembly of the mitotic spindle and attachment of cytoplasmic microtubules to kinetochores, which are crucial for proper partitioning of cellular materials into emerging daughter cells during cytokinesis. A significant amount of knowledge on cellular reorganization during mitosis had been reviewed (Wurzenberger & Gerlich, 2011). Here, we primarily focus on the function of PP2A in these critical processes.

Nuclear envelope breakdown and reassembly is tightly coordinated with mitotic phosphorylation and dephosphorylation (reviewed in Guttinger *et al.* (2009)). Nuclear envelope breakdown was facilitated by CDK1-dependent phosphorylation of lamin proteins and subsequent disassembly of the nuclear lamina (Peter *et al.*, 1990), and phosphorylation of nucleoporins-mediated disassembly of nuclear pore complexes (Laurell *et al.*, 2011). PP2A (Schmitz *et al.*, 2010) and its closely related PP1 (Thompson *et al.*, 1997) play a role

in nuclear envelope reassembly during mitotic exit with unclear mechanisms. It remains to be determined whether lamin and nucleoporins are the specific substrates of PP2A. Disassembly and reassembly of the Golgi apparatus is also driven by mitotic phosphorylation and dephosphorylation. While phosphorylation of Golgi matrix protein GM130 induces disassembly (Wei & Seemann, 2009), PP2A-mediated dephosphorylation of GM130 induces Golgi reassembly during mitotic exit, which involves PP2A-B55 α holoenzyme (Lowe *et al.*, 2000; Schmitz *et al.*, 2010).

Mitotic chromosomal assembly is regulated by condensin I and condensin II, which belongs to a class of conserved condensin complexes that play essential roles in mitotic chromosome condensation by collaborating with other chromosomal components (Hagstrom & Meyer, 2003; Jessberger, 2002). PP2A interacts with condensin II and plays an essential role in targeting condensin II to chromosomes (Takemoto *et al.*, 2009). Intriguingly, this process does not require the phosphatase activity of PP2A (Takemoto *et al.*, 2009). Chromatin decondensation requires PP1 and its regulatory subunits Repo-Man (recruits PP1 onto mitotic chromatin at anaphase protein) and PNUITS (phosphatase 1 nuclear targeting subunit) (Landsverk *et al.*, 2005; Vagnarelli *et al.*, 2006). The role of PP2A in this process is less characterized. Nonetheless, a recent study showed that a midzone-associated Aurora B gradient monitors chromosome position along the division axis and to prevent premature chromosome decondensation by retaining Condensin I until effective separation of sister chromatids is achieved (Afonso *et al.*, 2014). Both PP1 and PP2A phosphatases counteract this gradient and promoted chromosome decondensation (Afonso *et al.*, 2014).

Proper kinetochore–microtubule attachments are tightly controlled by Aurora B-mediated phosphorylation and PP2A/PP1-mediated dephosphorylation. Aurora B phosphorylates multiple substrates at the kinetochore to destabilize and correct erroneous kinetochore–microtubule attachments (Welburn *et al.*, 2010). While PP1 is considered the major phosphatase counteracting Aurora B (Carmena *et al.*, 2012), the PP2A-B56 α holoenzyme also plays a critical role in stabilizing kinetochore–microtubule attachments by counteracting Aurora B phosphorylation (Foley *et al.*, 2011). Pseudokinase BUBR1 seems to play a critical role in integration of kinase and phosphatase activities to ensure proper formation of stable kinetochore–microtubule attachments (Suijkerbuijk *et al.*, 2012). Phosphorylation of a conserved KARD domain in BUBR1 by PLK1 (polo-like kinase 1) promotes direct interaction of BUBR1 with the PP2A-B56 α phosphatase (Suijkerbuijk *et al.*, 2012), a potential mechanism for the recruitment of PP2A-B56 α to the inner kinetochore prior to microtubule attachment (Foley *et al.*, 2011). Removal of BUBR1 from mitotic cells or inhibition of PLK1 reduces PP2A-B56 α kinetochore binding (Suijkerbuijk *et al.*, 2012), suggesting that PLK1 and BUBR1 cooperate to stabilize kinetochore–microtubule interactions by regulating kinetochore localization of the PP2A-B56 α holoenzyme.

PP2A in spindle checkpoint, regulation of APC/C-CDC20 and mitotic exit

The rise in APC/C-CDC20 activity initiates mitotic exit by targeting several mitotic determinants for degradation, resulting in the formation and separation of two interphase daughter cells. APC/C is kept inactive by the spindle assembly checkpoint until all chromosomes attach to microtubules originating from opposite spindle poles [reviewed in

Musacchio & Salmon (2007)]. The early mitotic inhibitor 1 (Emi1) and 2 (Emi2) play a critical role in inhibition of APC/C, and PP2A-B56 holoenzymes was found to promote the inhibitory activity of Emi2 and maintain the spindle assembly checkpoint, which was antagonized by the activity of CDK1 (Tischer *et al.*, 2012). CDK1-mediated phosphorylation of APC/C inhibitory proteins primes its own inactivation. At mitotic exit, APC/C-CDC20 induces proteasomal destruction of cyclin B, and inactivates mitotic CDK1 (Sullivan & Morgan, 2007). Inactivation of CDK1 is expected to lead to reactivation of PP2A-B55 to mediate dephosphorylation of CDK1 substrates at mitotic exit.

Adenomatous polyposis coli (APC)/C-CDC20 also mediates the degradation of securin to initiate chromosome segregation. Securin inhibits the protease separase; removal of securin allows separase to cleave the sister chromatid cohesion 1 (SCC1) subunit of the cohesin complex (Sullivan & Morgan, 2007). Cohesin function is also regulated by PLK1 and PP2A. PLK1 promotes dissociation of cohesin from chromosome arms by phosphorylating the cohesin subunit SA2 during prometaphase (Sumara *et al.*, 2002). Shugoshin 1 recruits PP2A-B56 to protect SA2 against PLK1-mediated phosphorylation and thereby maintains a pool of persistent cohesion, and prevents premature separation of sister chromatids (Kitajima *et al.*, 2006; Tang *et al.*, 2006; Tanno *et al.*, 2010; Xu *et al.*, 2009).

With limited information on specific substrates targeted by diverse PP2A holoenzymes, the above knowledge likely merely represents a small fraction of PP2A function during mitosis. More questions need to be addressed regarding how PP2A holoenzyme activity is temporally and spatially controlled for tight regulation of numerous events during mitosis.

Concluding remarks

The cell cycle harbors complex and intricate processes and may be the most studied aspect in biology. This highlights its importance in understanding the origins of most of human disease and what can be done to intervene for therapeutic purposes. Great strides have been made in understanding the complex players of the cell cycle, and the importance of regulation by reversible phosphorylation cannot be underestimated.

The role of PP2A in regulating the cell cycle is only beginning to be investigated. It is involved in most major cell cycle initiation pathways as well as in regulating major checkpoints during cell cycle phase transitions. PP2A is implicated in dephosphorylating many more cell cycle pathway substrates than could be discussed here (Table 2), further highlighting its importance to properly functioning cells. The kinases involved in regulating the cell cycle typically exert their action through transcription-level changes and/or regulation of protein stability, whereas the cellular level of PP2A scaffold and catalytic subunits are stable throughout the cell cycle. Although PP2A expression differs from that leading to “canonical” kinase regulation, PP2A is one of the primary cell cycle regulating enzymes due to the dynamic nature of its holoenzyme assembly, activation and inhibition. As discussed, it is a major target in several key pathways, both for protein activation and inactivation, and it is frequently targeted for inhibition due to its antagonistic effects in these pathways. Although kinases may have taken center stage in the study of cell cycle

regulation, the intricate connectivity between these kinases and PP2A shows its importance in the tight regulation of these processes.

In addition to its wide involvement in cellular processes, PP2A is a complex group of enzymes, and its assembly and activity are highly regulated making it challenging to study. No PP2A-substrate binding consensus sequence has been identified yet, indicating that structural and biochemical information is required to understand the mechanisms by which PP2A regulates substrate dephosphorylation. Currently, high-throughput assays are being explored to identify substrates and characterize protein–protein interactions significantly faster than traditional methods. These large scale assays combined with structural and biochemical characterization will provide an unprecedented amount of information to the PP2A field and possibly identify new targets of cell cycle regulation. These new targets may be incredibly useful in developing drugs or biomarkers for preventing, diagnosing or treating human disease, and more refined knowledge on existing PP2A-substrate interactions may help improve current treatments.

Acknowledgments

Declaration of interest

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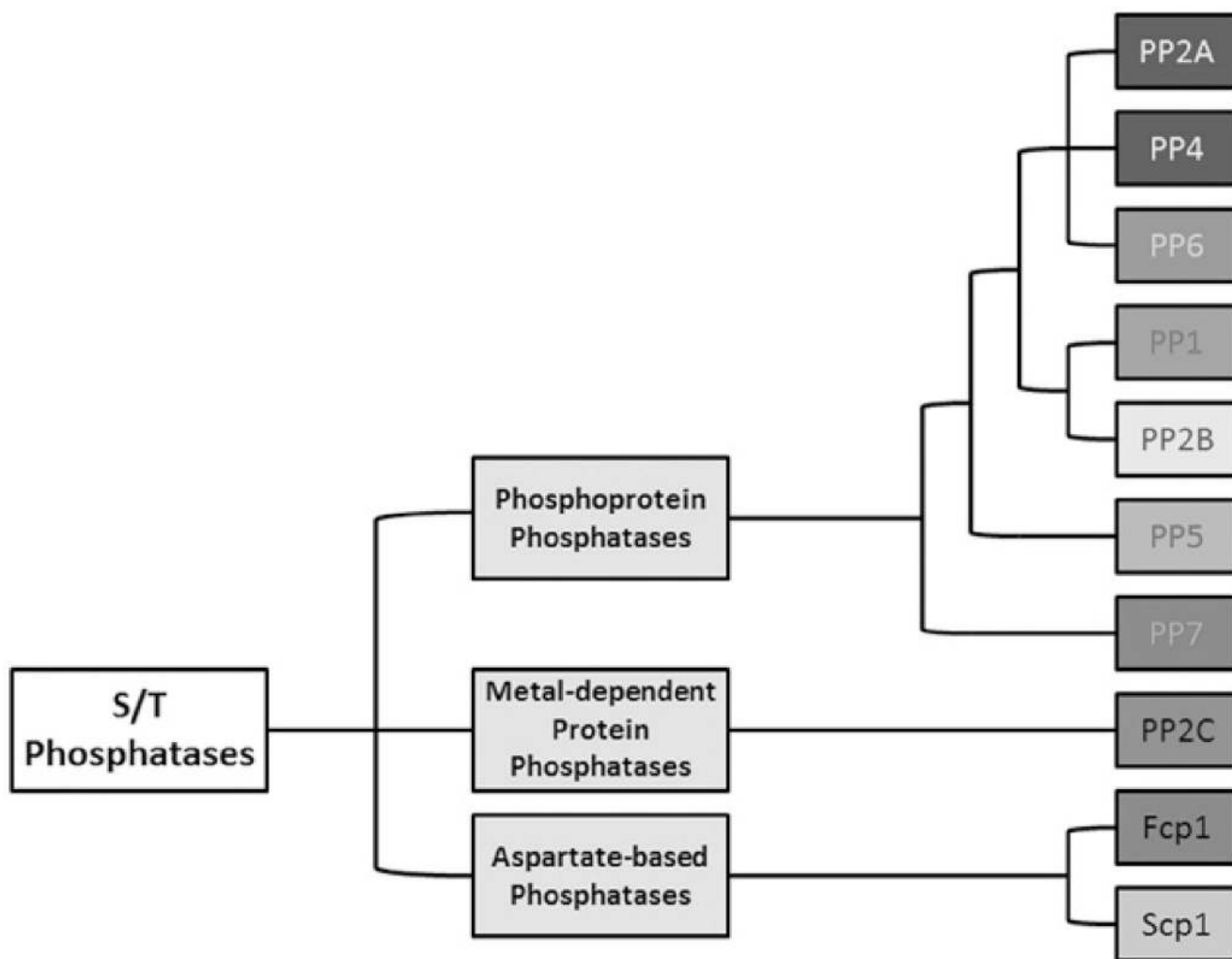


Figure 1. Serine/threonine phosphatases are classified based on biochemical mechanism. They are divided into three families, the aspartate-based phosphatases, the metal-dependent protein phosphatases and the phosphoprotein phosphatases. The phosphoprotein phosphatases have similar active site configurations and require catalytic metal ions in the active site. PP2A is a member of this family. Adapted from Stanevich (2013). (see colour version of this figure at www.informahealthcare.com/bmg).

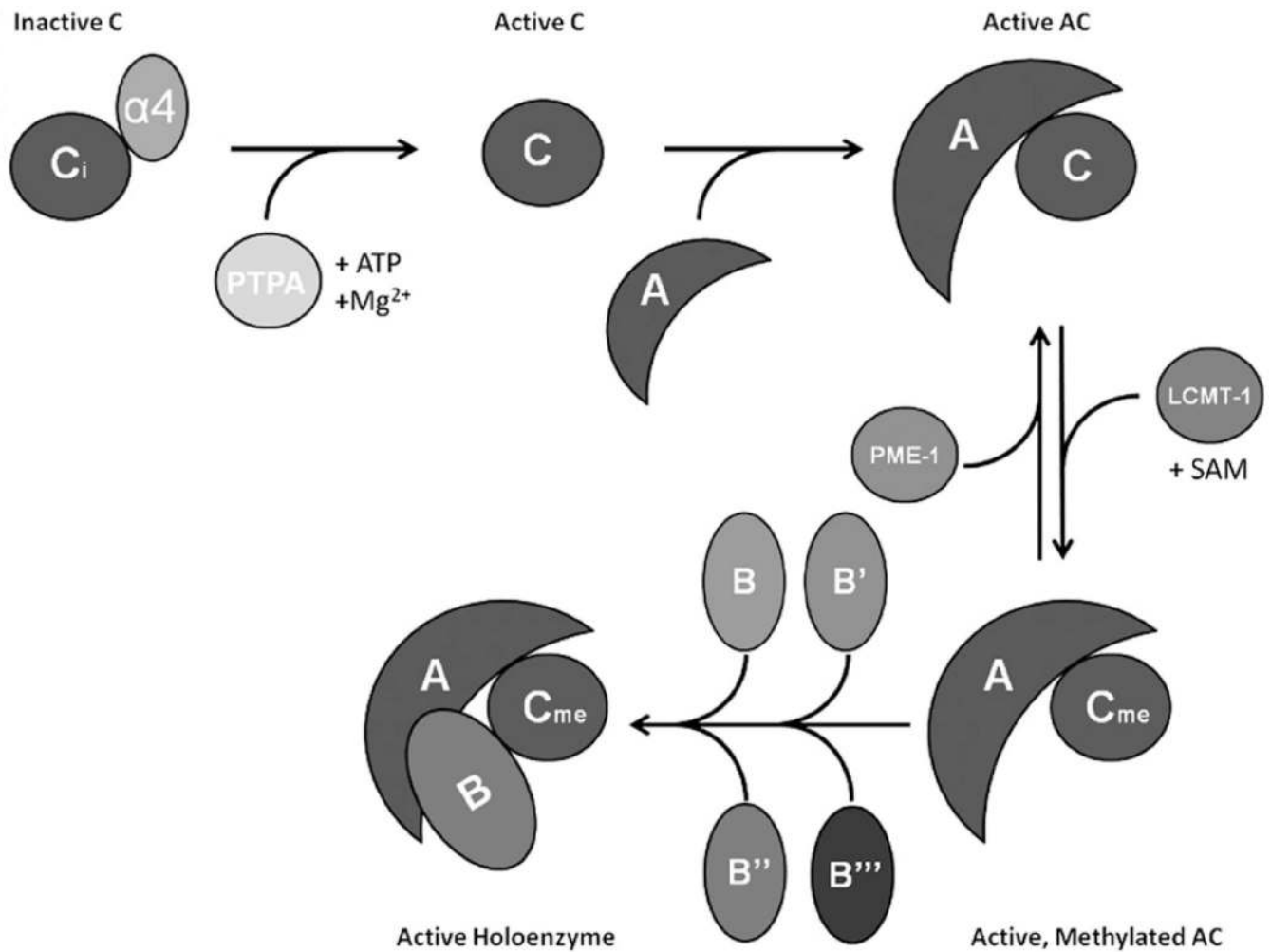


Figure 2.

PP2A biogenesis and holoenzyme assembly is regulated by unique factors. $\alpha 4$ protects inactive PP2Ac from ubiquitination by MID1. Activating metal ions are loaded by PTPA, and active PP2Ac binds to the scaffold subunit (A). The C-terminal tail of PP2Ac can be methylated by LCMT-1 and reversed by PME-1. Active, methylated PP2A-AC can then form holoenzymes with one B subunit. These available B subunits are divided into four families: B, B', B'' and B''', each with unique characteristics and regulation. (see colour version of this figure at www.informahealthcare.com/bmg).

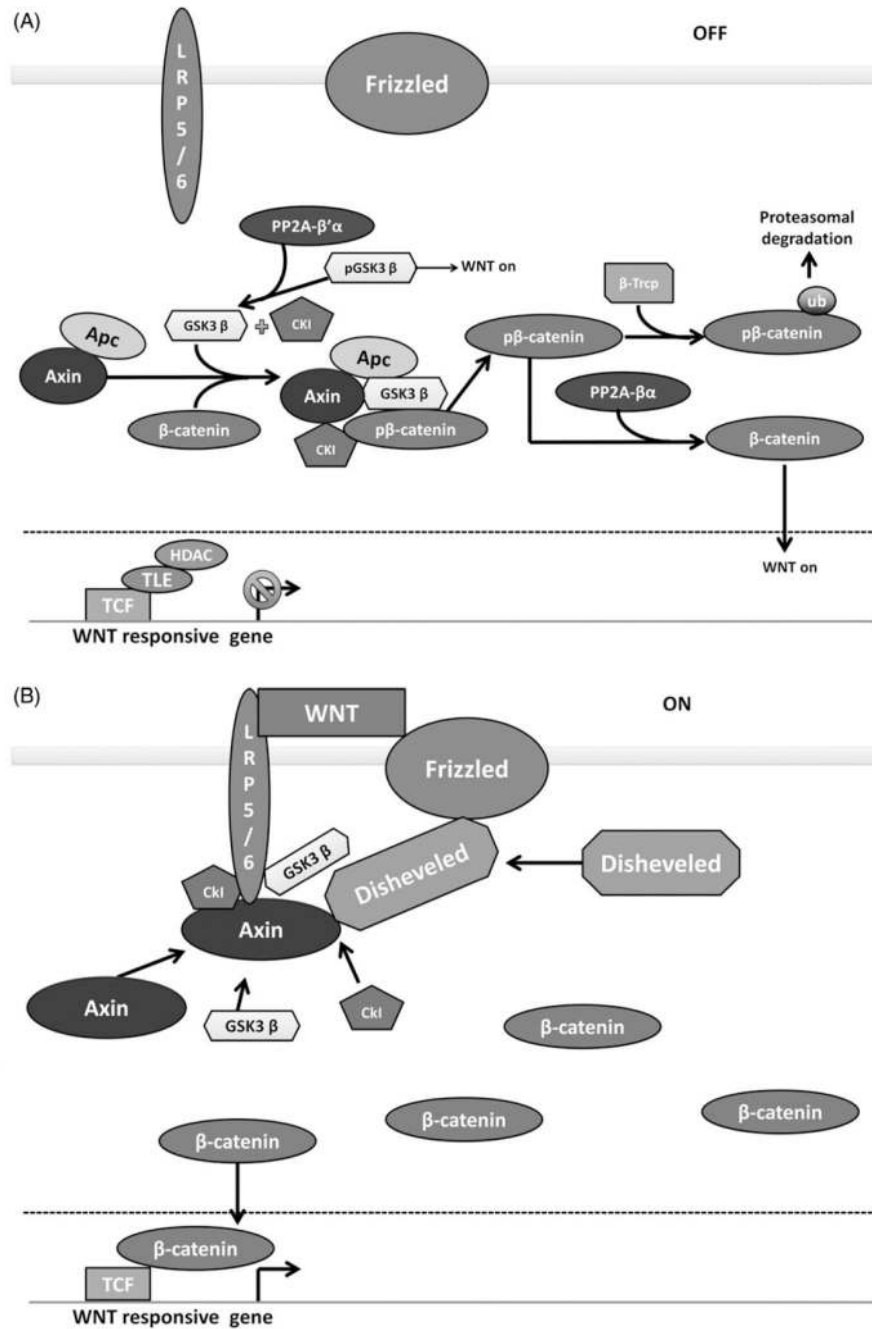


Figure 3. PP2A positively and negatively regulates the Wnt signaling pathway. (A) Wnt OFF. In the absence of Wnt signaling, a complex of Axin, Apc, GSK3 β and CK1 phosphorylate β -catenin, targeting it for proteasomal degradation. PP2A-B' α promotes β -catenin degradation by removing an inhibitory phosphorylation on GSK3 β . PP2A-B α can directly dephosphorylate β -catenin, promoting the activation of wnt responsive genes. (B) Wnt signaling ON. In the presence of Wnt ligand, Wnt receptors LRP5/6 and frizzled sequester the Axin, GSK3 β and CK1, preventing the phosphorylation of β -catenin. β -Catenin

accumulates and translocates to the nucleus, promoting the transcription of Wnt responsive genes. Figure adapted from MacDonald *et al.* (2009). (see colour version of this figure at www.informahealthcare.com/bmg).

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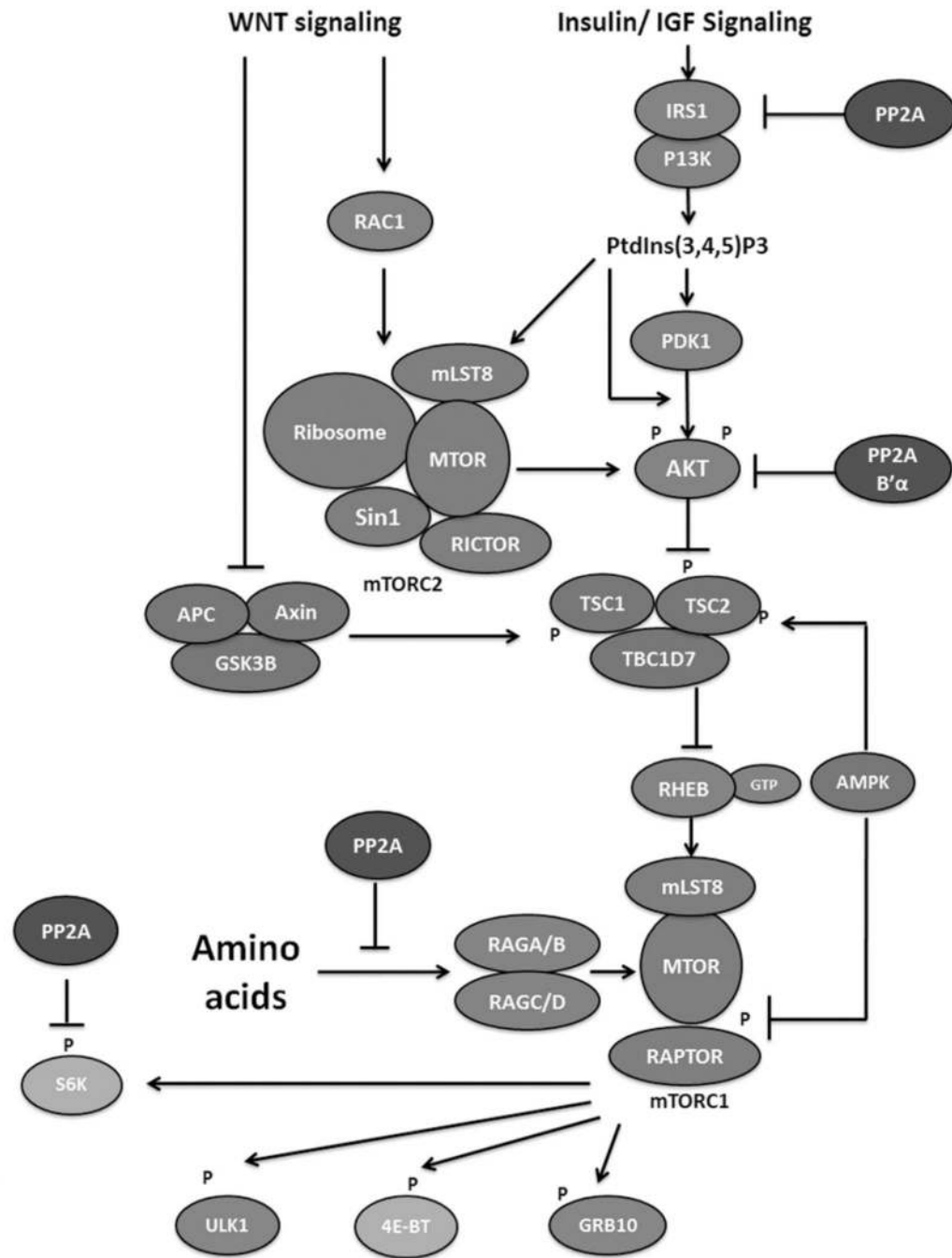


Figure 4. PP2A negatively regulates the mTOR signaling pathway. The mTOR complexes are colored blue. Proteins involved in mTOR inhibition and activation are colored red and green, respectively with PP2A in purple. Downstream factors inhibiting and stimulating growth are colored magenta and teal, respectively. Growth factors stimulate the mTOR pathway via inhibiting the function of the TSC complex that inhibits mTOR activation. Wnt signaling can inhibit the TSC complex or directly stimulate mTORC2. Amino acids can also stimulate mTOR activity. PP2A inhibits the mTOR pathway by inhibiting IRS1 in the insulin

signaling pathway or MAP4K3 in the amino acid pathway, or by inhibiting AKT function. PP2A can also reverse mTOR phosphorylation of S6K. Figure adapted from Shimobayshi & Hall (2014). (see colour version of this figure at www.informahealthcare.com/bmg).

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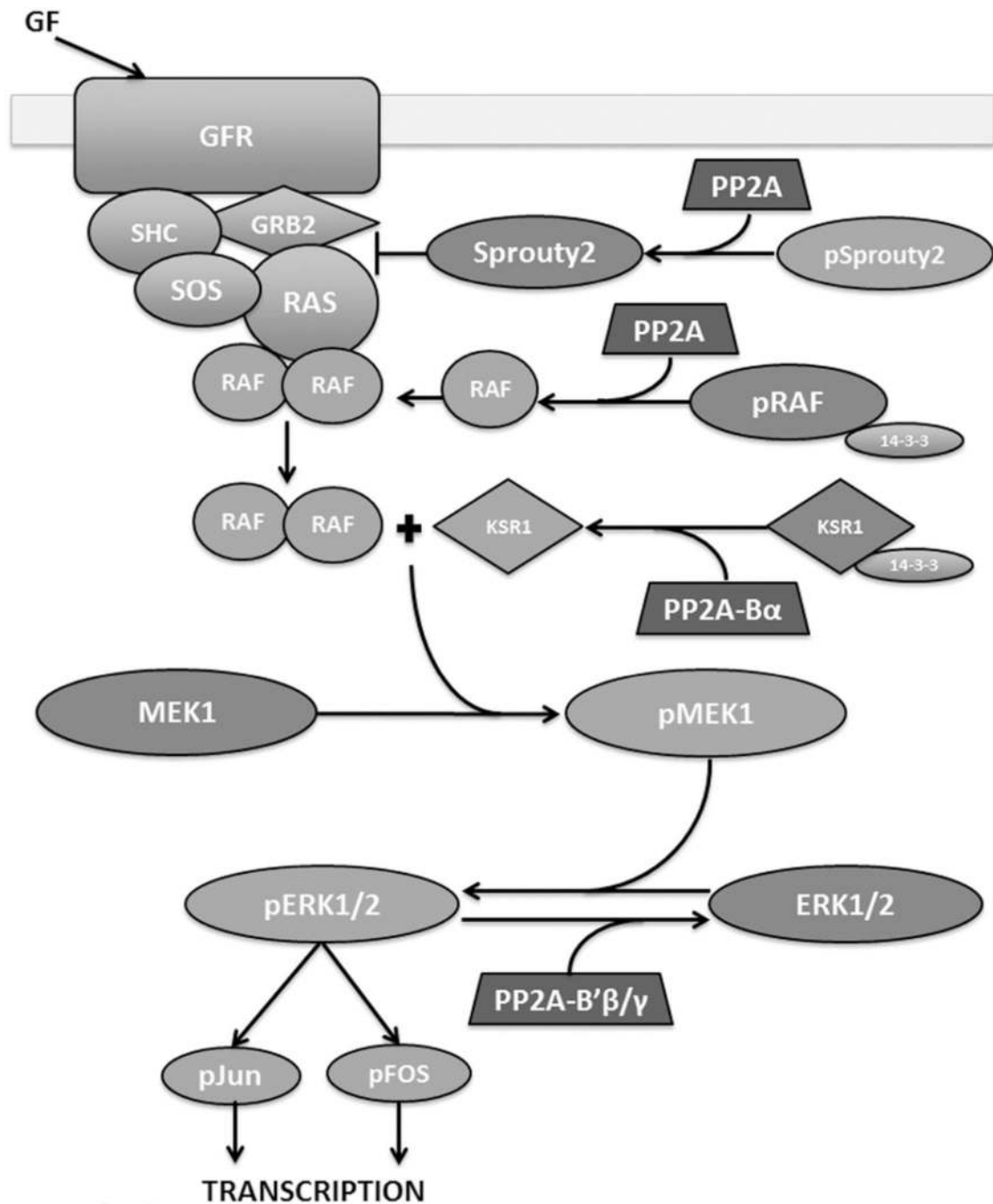


Figure 5.

PP2A positively and negatively regulates the MAPK signaling pathway. Growth factors stimulate a complex of proteins: SHC, GRB2 and SOS to assemble on a growth factor receptor. This complex activates Ras which starts a signal cascade from activation of Raf, to activation of MEK, ERK and eventually the transcription factors that activate the transcription of growth related genes. PP2A can activate Raf by dephosphorylating S259 and causing 14–3–3 release. PP2A-B α dephosphorylates S392 of KSR1, which leads to dissociation of 14–3–3 from KSR1, essential for MEK1 activation. PP2A negatively

regulates MAPK upstream by activating Sprouty2, which inhibits GRB2 and subsequent RAS complex formation. PP2A-B β /B γ can directly dephosphorylate ERK1/2 downstream of the signaling cascade, thereby inactivating it. Factors promoting cell division are shown in green and those opposing cell division are shown in red. Figure adapted from McCubrey *et al* (2007). (see colour version of this figure at www.informahealthcare.com/bmg).

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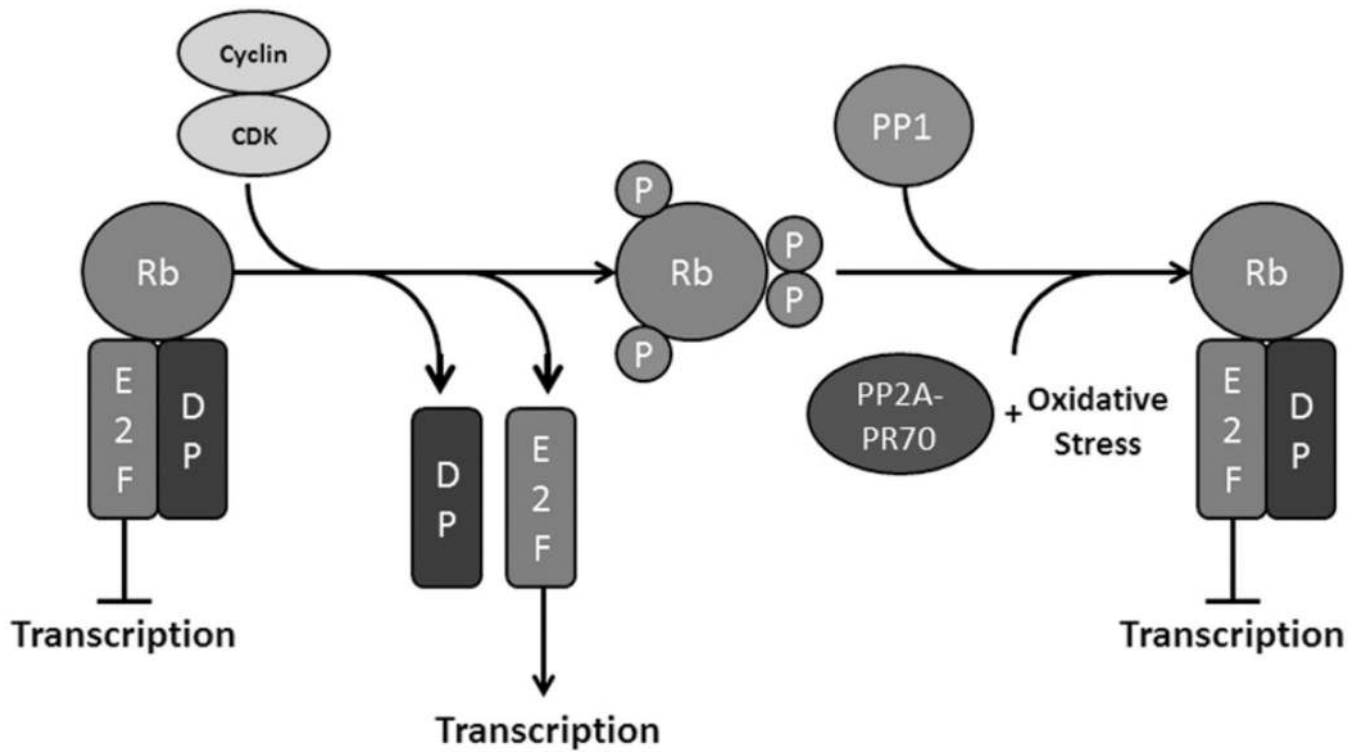


Figure 6.

Rb phosphorylation promotes transcription of E2F responsive genes. Rb normally binds E2F transcription factors and their dimerization partners. When phosphorylated by cyclin/CDK heterodimers, Rb loses affinity for E2F and free E2F is allowed to promote transcription. Rb is dephosphorylated at the end of mitosis, allowing reassociation with E2F. Normally, PP1 dephosphorylates Rb at the end of mitosis, but PP2A-PR70 can dephosphorylate Rb under oxidative stress conditions. (see colour version of this figure at www.informahealthcare.com/bmg).

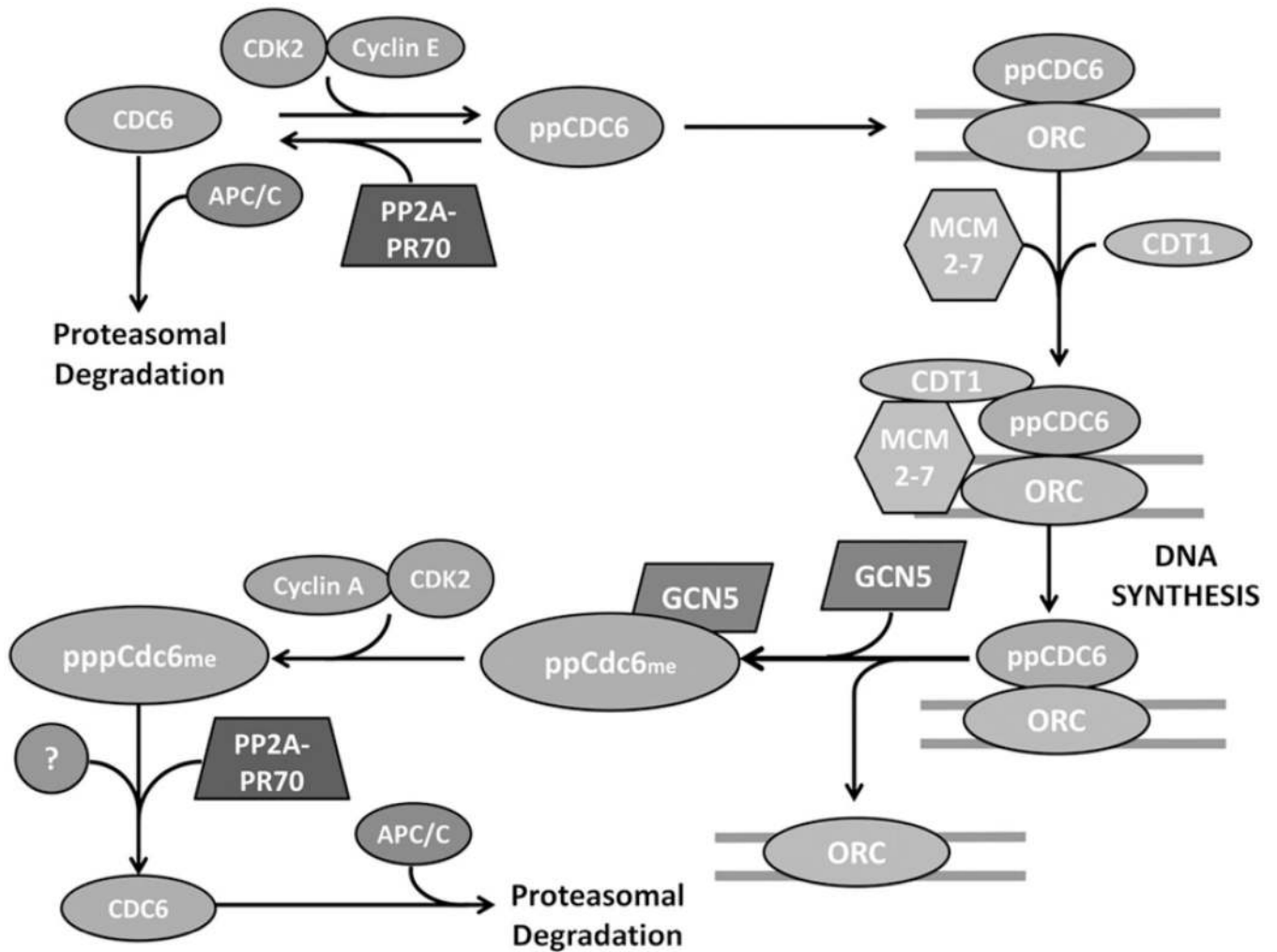


Figure 7.

Cdc6 is necessary for assembly of the pre-replication complex and subsequent DNA synthesis. In G₀, Cdc6 is ubiquitinated by the anaphase promoting complex/cyclosome (APC/C) and degraded by the proteasome. In G₁, Cyclin E/CDK2 phosphorylates Cdc6 on S54 and S74, protecting it from degradation. Cdc6 is translocated into the nucleus where it binds the origin recognition complex and is required to recruit Cdt1 and MCM2–7 and form the prereplication complex. After firing of the origins, Cdc6 is methylated by GCN5 causing its dissociation from the ORC. Cdc6 is then phosphorylated on S106 by Cyclin A/CDK2 and translocated to the cytoplasm. PP2A-PR70 is thought to dephosphorylate Cdc6 either at this point in G₂ and/or in G₁, ensuring Cdc6 destruction and regulating DNA synthesis. Figure adapted from Mumby (2009). (see colour version of this figure at www.informahealthcare.com/bmg).

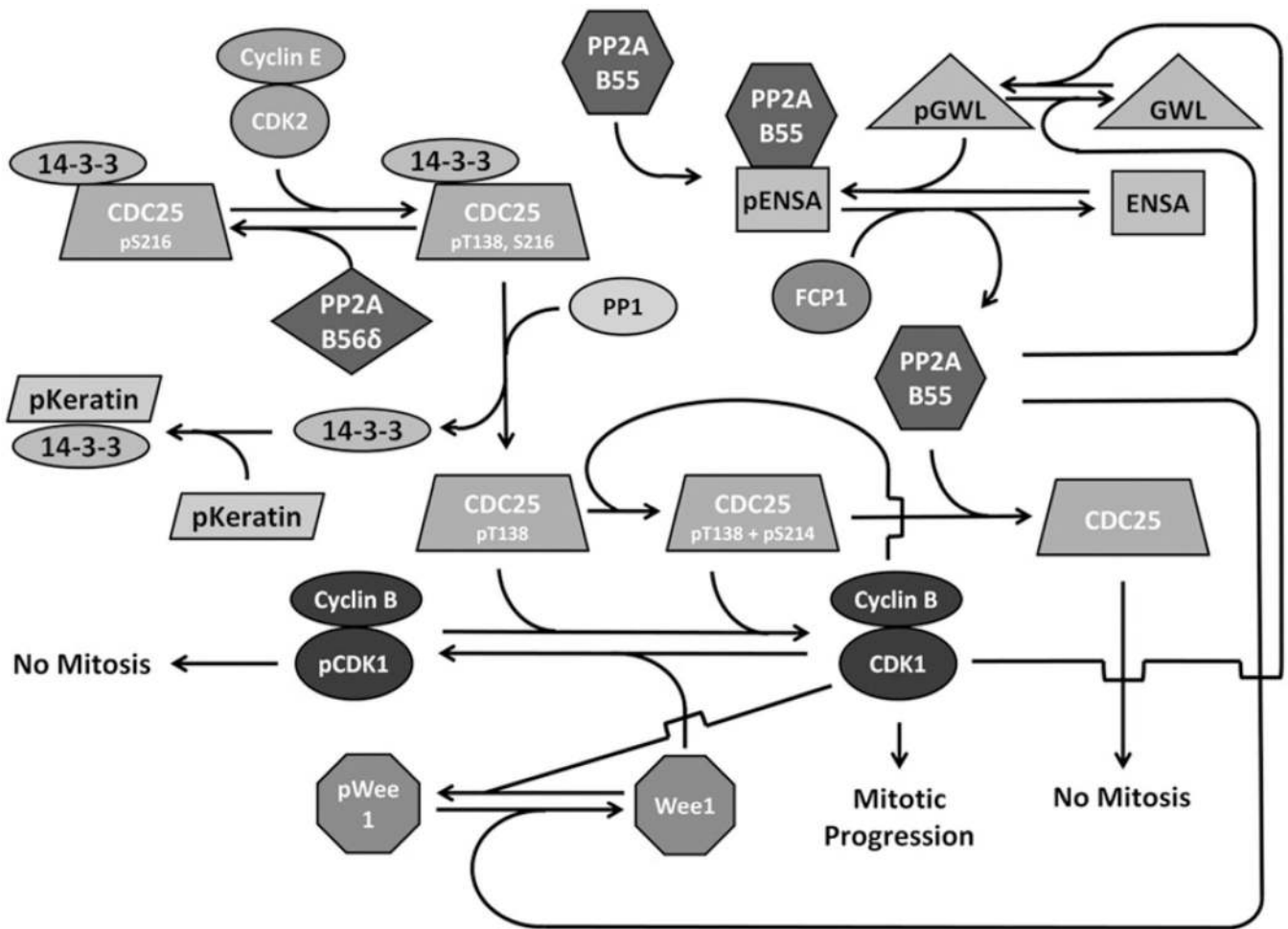


Figure 8.

PP2A negatively regulates the cell cycle through CDC25 and Wee1. In G2, Greatwall, Fcp and Wee1 are dephosphorylated, keeping PP2A-B55 active and CDK1 inactive. CDC25 is phosphorylated at S216, allowing 14-3-3 association, holding it inactive. At the transition from G2 to M, CDC25 is phosphorylated at T138, weakening 14-3-3 binding and allowing dissociation with subsequent binding to a phospho-keratin pool. The now exposed S216 can be dephosphorylated by PP1 activating CDC25. Active CDC25 dephosphorylates CDK1 at T14 and Y15 thereby activating it. The active CDK1 can then phosphorylate several substrates required for mitotic progression. In addition, CDK1 participates in several positive feedback loops. It phosphorylates Wee1, preventing direct inactivation, and it phosphorylates CDC25 at S214, increasing its affinity for PP1 and allowing for more CDC25 activation. Furthermore, CDK1 can prevent CDC25 inactivation by PP2A-B55 by phosphorylating Greatwall, which in turn phosphorylates ENSA, which binds to PP2A-B55 and keeps it inactive. CDK1 also phosphorylates FCP1, preventing it from dephosphorylating ENSA and releasing PP2A-B55. Figure adapted from Johnson & Kornbluth (2012) and Hegarat *et al* (2014). (see colour version of this figure at www.informahealthcare.com/bmg).

Table 1

A summary of PP2A subunit nomenclature.

PP2A subunit family	Protein name/isoform	Other names
A	A α	PR65 α
	A β	PR65 β
B/PR55	B α	B55 α /PR55 α
	B β 1	B55 β 1/PR55 β 1
	B β 2	B55 β 2/PR55 β 2
	B γ	B55 γ /PR55 γ
	B δ	B55 δ /PR55 δ
B'/PR61	B α	B56 α /PR61 α
	B β	B56 β /PR61 β
	B' γ 1	B56 γ 1/PR61 γ 1
	B' γ 2	B56 γ 2/PR61 γ 2
	B' γ 3	B56 γ 3/PR61 γ 3
	B' δ	B56 δ 1/PR61 δ
B''/PR72	B'' α	PR130
	B'' α	PR72
	B'' β	PR70
	B'' γ	G5PR
B'''/Striatin	B'''	Striatin
C	C α	PP2A α
	C β	PP2A β

There is a variety of abbreviations used for protein subunits in the PP2A field. Many regulatory subunits have splicing variants, such as B β and B' γ . Note that not all of them are shown. Table adapted from Van Kanegan & Strack (2009).

Table 2

PP2A-interacting proteins identified by literature and database searches. Reviews, Pubmed and Biogrid (Chatr-Aryamontri *et al.*, 2015) results are presented.

Interacting proteins	B subunit	PhosphoSite	Interaction boundary	References
ADCY8	-	-	-	(Crossthwaite <i>et al.</i> , 2006)
ADRA1A	-	-	-	(Krueger <i>et al.</i> , 1997)
AKAP9	PR130	-	-	(Takahashi <i>et al.</i> , 1999)
AKT	B α	T308	-	(Kuo <i>et al.</i> , 2008)
APC	B', B''	bCatenin, -	302-625, 188-774	(Breitman <i>et al.</i> , 2008, Galea <i>et al.</i> , 2001)
AP1M1	B α	T156	-	(Ricotta <i>et al.</i> , 2008)
APP	B' γ , B' ϵ	-	-	(Olah <i>et al.</i> , 2011)
AR	-	-	-	(Yang <i>et al.</i> , 2007)
ARL2	B α	-	-	(Shern <i>et al.</i> , 2003)
ATM	-	S1981	2427-2841	(Goodarzi <i>et al.</i> , 2004)
ATR	-	-	-	(Kim <i>et al.</i> , 1999)
ATXN7L2	PR72	-	-	(Lim <i>et al.</i> , 2006)
AURKA	-	S51	46-56	(Horn <i>et al.</i> , 2007)
AXIN1	B' α	-	595-726	(Arnold <i>et al.</i> , 2009, Yamamoto <i>et al.</i> , 2001)
BANF1-ANKLE4	B α	-	59-938	(Asencio <i>et al.</i> , 2012)
ARRB2	-	-	-	(Beaulieu <i>et al.</i> , 2005)
BAX	-	S184	-	(Xin & Deng, 2006)
BAZ	B'	S1085	-	(Krahn <i>et al.</i> , 2009)
BCL2	B' γ	T69, S70, S87	-	(Lin <i>et al.</i> , 2006), (Ruvolo <i>et al.</i> , 2008)
BEST1	-	-	-	(Marmorstein <i>et al.</i> , 2002)
BLNK	B γ	-	-	(Oellerich <i>et al.</i> , 2011)
BRCA1	B' γ	-	-	(Woods <i>et al.</i> , 2012)
BUBR1	B'	-	630-720	(Xu <i>et al.</i> , 2013)
CACNA1C	B α , B β , PR70	S1928	1927-2029	(Hall <i>et al.</i> , 2006)
CACNA1S	B α	-	-	(Kristensen <i>et al.</i> , 2012)
CAPN1	-	-	-	(Hall <i>et al.</i> , 2006)
CAMK4	-	-	-	(Westphal <i>et al.</i> , 1998)
CAMK2B	-	T253	-	(Hoffmann <i>et al.</i> , 2005)
CPD	-	-	-	(Varlamov <i>et al.</i> , 2001)
CAS	-	-	-	(Yokoyama & Miller, 2001)
CASP3	-	-	-	(Alvarado-Kristensson & Andersson, 2005)
CBX1	-	-	-	(Nozawa <i>et al.</i> , 2010)
CCNG1	B' α	-	-	(Okamoto <i>et al.</i> , 1996,2002)
CCNG2	B' β , B' γ	-	-	(Bennin <i>et al.</i> , 2002)
CCT2	B γ	-	-	(Glatter <i>et al.</i> , 2009)
CDCA2 (repoman)	B' α	S893	586-595	(Qian <i>et al.</i> , 2013)
CDC25	B'	T138	-	(Lammer <i>et al.</i> , 1998, Margolis <i>et al.</i> , 2003,2006a,b)
CDC6	PR70	S54/S74	49-90	(Davis <i>et al.</i> , 2008, Petersen <i>et al.</i> , 1999,

Interacting proteins	B subunit	PhosphoSite	Interaction boundary	References
				Wlodarchak <i>et al.</i> , 2013)
CDH1 (e-cadherin)	–	–	–	(Gotz <i>et al.</i> , 2000)
CDK16	B α	–	–	(Varjosalo <i>et al.</i> , 2013a)
CDK 4	–	–	–	(Sablina <i>et al.</i> , 2007)
CDK5R1	B' δ	–	–	(Louis <i>et al.</i> , 2011)
CDK9	–	–	–	(Ammosova <i>et al.</i> , 2005)
CFTR	–	–	–	(Vastiau <i>et al.</i> , 2005)
CHEK1	–	S317 & S345	–	(Leung-Pineda <i>et al.</i> , 2006)
CIP2A	–	–	–	(Junttila <i>et al.</i> , 2007)
CFL1	–	–	–	(Samstag & Nebl, 2003)
GJA1	–	–	–	(Kanemitsu <i>et al.</i> , 1998, Meilleur <i>et al.</i> , 2007)
CSNK1E	–	–	–	(Varjosalo <i>et al.</i> , 2013b)
CTLA4	B' α	–	151–159	(Baroja <i>et al.</i> , 2002)
CTNNB1 (β -Catenin)	B', B α	S33, S37, T41, S45, S552, S675	APC, Mult.	(Seeling <i>et al.</i> , 1999, Zhang <i>et al.</i> , 2009)
CUL3	B' β	–	315–374	(Bennett <i>et al.</i> , 2010, Oberg <i>et al.</i> , 2012)
CXCR2	–	–	–	(Fan <i>et al.</i> , 2001)
DARPP32	PR72	T75	–	(Ahn <i>et al.</i> , 2007b)
DLG4	B' δ	–	–	(Arbuckle <i>et al.</i> , 2010)
DNML1/DRP1	B β 2	S656	–	(Merrill <i>et al.</i> , 2013)
E4orf3 [adenovirus]	–	–	–	(Shtrichman <i>et al.</i> , 1999)
EGFR	PR130, B' ϵ	–	–	(Foerster <i>et al.</i> , 2013, Zwaenepoel <i>et al.</i> , 2010)
EIF1AK2	B' α	–	–	(Xu & Williams, 2000)
EIF4EBP1	B α	–	–	(Bishop <i>et al.</i> , 2006)
ELAVL1	B' ϵ	–	–	(Abdelmohsen <i>et al.</i> , 2009)
ERAL1	–	S18	–	(Keen <i>et al.</i> , 2005)
ETF1	–	–	–	(Lechward <i>et al.</i> , 1999)
ERK1/ERK2	B' β , B' γ	T202/T185	IEK-1	(Letourneux <i>et al.</i> , 2006)
ESPL1 (separase)	B' α - ϵ	–	1419–1474	(Holland <i>et al.</i> , 2007)
FAM107A	B α	–	–	(Ewing <i>et al.</i> , 2007)
FBXO43	–	–	319–375	(Wu <i>et al.</i> , 2007)
FMRP	–	–	–	(Narayanan <i>et al.</i> , 2007)
GNA12	–	–	–	(Zhu <i>et al.</i> , 2004)
GNB2L1	–	–	138–317	(Kiely <i>et al.</i> , 2006)
GRIA1	–	–	–	(Mao <i>et al.</i> , 2005)
GRB2	B α	–	–	(Bisson <i>et al.</i> , 2011)
GRIN2A	–	–	–	(Chan & Sucher, 2001)
GRK5	B α	–	–	(Wu <i>et al.</i> , 2012)
GSK3B	B' δ	S9	–	(Liu & Eisenman, 2012)
H2AX	–	–	–	(Chowdhury <i>et al.</i> , 2005)
HAND1	B' δ	–	150–216	(Firulli <i>et al.</i> , 2003)
HAND2	B' δ	–	–	(Firulli <i>et al.</i> , 2003)
HCP6	B	–	–	(Yeong <i>et al.</i> , 2003)

Interacting proteins	B subunit	PhosphoSite	Interaction boundary	References
HDAC4	B α	S298	1–289	(Paroni <i>et al.</i> , 2008)
HDAC5	B α	–	–	(Greco <i>et al.</i> , 2011, Joshi <i>et al.</i> , 2013)
HDM2	CyG	S166	400–489	(Okamoto <i>et al.</i> , 2002)
HOX11	–	–	–	(Kawabe <i>et al.</i> , 1997)
HRX	–	–	–	(Adler <i>et al.</i> , 1997)
HSF2	–	–	–	(Xing <i>et al.</i> , 2007)
HTR1A	–	–	–	(Bauman <i>et al.</i> , 2000)
IER3	–	–	–	(Letourneux <i>et al.</i> , 2006)
IKBKB	–	S77 & S181	121–179	(Kray <i>et al.</i> , 2005, Li <i>et al.</i> , 2006)
IKBKG	–	S68	–	(Fu <i>et al.</i> , 2003, Palkowitsch <i>et al.</i> , 2008)
IL6ST	–	S782	–	(Mitsubishi <i>et al.</i> , 2005)
IQGAP1	–	–	–	(Takahashi & Suzuki, 2006)
IRAK1	–	–	–	(Dobierzewska <i>et al.</i> , 2011)
JAK2	–	–	–	(Fuhrer & Yang, 1996)
JNK	–	–	–	(Shanley <i>et al.</i> , 2001)
KCNQ2	B' γ	–	E12–14	(Borsotto <i>et al.</i> , 2007)
KRT8	–	–	–	(Tao <i>et al.</i> , 2006)
KRT18	–	–	–	(Tao <i>et al.</i> , 2006)
KSR1	B α	S392	249–320	(Ory <i>et al.</i> , 2003)
LATS2	B α	–	–	(Woods <i>et al.</i> , 2012)
LNX1	–	–	–	(Guo <i>et al.</i> , 2012)
MAPK14	–	–	–	(Alvarado-Kristensson & Andersson, 2005)
MASTL (greatwall)	B α	T194	–	(Hegarati <i>et al.</i> , 2014)
MDM2	B' α /CyG	T216	100–280, 400–489	(Okamoto <i>et al.</i> , 2002)
MEK3	α 4	T193	–	(Prickett & Brautigam, 2007)
MEKK3	B α , B δ	S526	–	(Fritz <i>et al.</i> , 2006)
MET	–	S985	–	(Hashigasako <i>et al.</i> , 2004)
MID1	–	–	–	(Short <i>et al.</i> , 2002)
MKK4	–	–	–	(Avdi <i>et al.</i> , 2002)
MLH1	B β &B δ	–	–	(Cannavo <i>et al.</i> , 2007)
MTOR	B α	–	–	(Peterson <i>et al.</i> , 1999)
MYC (cMYC)	B' α	S62	40–179	(Arnold & Sears, 2006)
NDRG1	B α	–	–	(Tu <i>et al.</i> , 2007)
NEK1	B' α	S109	1–267	(Surpili <i>et al.</i> , 2003)
NHE3	B' δ	–	651–839	(Bobulescu <i>et al.</i> , 2010)
NKCC1	–	–	–	(Liedtke <i>et al.</i> , 2005)
NKD1	PR72	–	–	(Creighton <i>et al.</i> , 2005, 2006)
NM23H2	–	–	–	(Chen <i>et al.</i> , 2008)
NOD2	B' ϵ	–	–	(Nimmo <i>et al.</i> , 2011)
NOTCH1	B α	–	–	(Yatim <i>et al.</i> , 2012)
NR3A	–	–	–	(Ma & Sucher, 2004)
NRF1	B' γ	–	–	(Satoh <i>et al.</i> , 2013)

Interacting proteins	B subunit	PhosphoSite	Interaction boundary	References
NTRK1	B'β & B'δ	–	–	(Van Kanegan & Strack, 2009)
OCLN	–	–	–	(Seth <i>et al.</i> , 2007)
OSBP	–	–	–	(Wang <i>et al.</i> , 2005)
PACS1	–	S278	–	(Scott <i>et al.</i> , 2003)
PAK1	–	–	–	(Westphal <i>et al.</i> , 1999)
PAK3	–	–	–	(Westphal <i>et al.</i> , 1999)
PDE4D/AKAP1	B'δ	–	2083–2319	(Dodge-Kafka <i>et al.</i> , 2010)
PER3	–	–	–	(Sathyanarayanan <i>et al.</i> , 2004)
PIM1	B'β	–	70–139	(Ma <i>et al.</i> , 2007)
PIN1	B'β	–	–	(Huang <i>et al.</i> , 2001, Michniewicz <i>et al.</i> , 2007)
PKR	B'α	B'α	B'α	(Xu & Williams, 2000)
POLA2	–	–	–	(Dehde <i>et al.</i> , 2001)
PPFIA1	B'δ	–	–	(Rual <i>et al.</i> , 2005)
PRKAR1A	–	–	–	(Ahn <i>et al.</i> , 2007a)
PTPN14	–	–	–	(Wang <i>et al.</i> , 2012)
PTTG1 (Securin)	–	–	–	(Gil-Bernabe <i>et al.</i> , 2006)
PXN (paxillin)	B'γ1/2	–	–	(Ito <i>et al.</i> , 2000)
RAF1	Bα/δ	S259	–	(Adams <i>et al.</i> , 2005)
RALA	Aβ	S183, S184	–	(Sablina <i>et al.</i> , 2007)
RB1	PR70	T826	792–928	(Lees <i>et al.</i> , 1991, Magenta <i>et al.</i> , 2008)
RBL1 (p107)	PR59	Mult.	–	(Voorhoeve <i>et al.</i> , 1999)
REC8	B'-sgo	Mult.	–	(Ishiguro <i>et al.</i> , 2010)
RELA	A	S536	1–155, 354–551	(Fuhrer & Yang, 1996, Li <i>et al.</i> , 2006, Yang <i>et al.</i> , 2001)
REV1	Bα & Bδ	–	–	(Naji <i>et al.</i> , 2012)
RHEB	–	–	–	(Lee <i>et al.</i> , 2007)
RHOB	–	–	–	(Lee <i>et al.</i> , 2007)
RPS6KB1	–	–	–	(Westphal <i>et al.</i> , 1999)
RRAS	–	–	–	(Lee <i>et al.</i> , 2007)
RRN3	Bα	S44	–	(Mayer <i>et al.</i> , 2004)
RSA1 & RSA2	–	–	–	(Schlaitz <i>et al.</i> , 2007)
RUNX2	–	–	–	(Rajgopal <i>et al.</i> , 2007)
SET	–	–	–	(Li <i>et al.</i> , 1996)
SG2NA	–	–	–	(Moreno <i>et al.</i> , 2000)
SHC	–	Y317	–	(Ugi <i>et al.</i> , 2002)
SLC6A2	–	–	–	(Sung <i>et al.</i> , 2005)
SMAD3	–	–	1–232	(Heikkinen <i>et al.</i> , 2010)
SMAD9	B'ε	–	–	(Colland <i>et al.</i> , 2004)
SMURF1	Bδ	–	–	(Xie <i>et al.</i> , 2013)
SOX2	B'γ	–	–	(Cox <i>et al.</i> , 2013)
SP1	–	T739	–	(Chuang <i>et al.</i> , 2012)
SPHK1	B'α	S225	451–470	(Pitman <i>et al.</i> , 2011)

Interacting proteins	B subunit	PhosphoSite	Interaction boundary	References
SPRY1	A	S112, S115	50–60	(Lao <i>et al.</i> , 2007)
SRC	B γ	S12	–	(Eichhorn <i>et al.</i> , 2007)
STAT5	–	–	–	(Yokoyama <i>et al.</i> , 2001)
STE20	–	–	–	(Liedtke <i>et al.</i> , 2005)
STK24	B $'''$	T178, T182	–	(Gordon <i>et al.</i> , 2011)
SUMO1	–	–	–	(Grant, 2010)
TAU	B α	Mult.	197–259, 265–328	(Xu <i>et al.</i> , 2008)
TAX	–	–	–	(Fu <i>et al.</i> , 2003)
TBC1D3	B' γ	S6K	–	(Wainszelbaum <i>et al.</i> , 2012)
TCEAL1	B α & B δ	–	–	(Sowa <i>et al.</i> , 2009)
TGFBR1	B α	–	–	(Griswold-Prenner <i>et al.</i> , 1998)
TH	B' β	S19, S31, S40	R37 & R38	(Saraf <i>et al.</i> , 2010)
TIP	–	–	–	(McConnell <i>et al.</i> , 2007)
TOM22	B β	–	–	(Dagda <i>et al.</i> , 2005)
TOP1	B β	–	–	(Trzcinska <i>et al.</i> , 2002)
TP53 (p53)	–, B' γ 1/3	S37, T55	–	(Dohoney <i>et al.</i> , 2004, Li <i>et al.</i> , 2007)
TRAF2	B' γ	T117	272–501	(Li <i>et al.</i> , 2006)
TSC2	–	–	–	(Lee <i>et al.</i> , 2007)
TTP	–	–	–	(Sun <i>et al.</i> , 2007)
UBD	–	–	–	(Aichem <i>et al.</i> , 2012)
UBR5	B γ	–	–	(Chen <i>et al.</i> , 2013)
UPF1	–	–	–	(Ohnishi <i>et al.</i> , 2003)
VIM	B α	–	–	(Turowski <i>et al.</i> , 1999)
VPU [HIV]	B' ϵ	–	–	(Jager <i>et al.</i> , 2012)
ZRANB1	B $'''$	–	–	(Tran <i>et al.</i> , 2013)

The first row is the abbreviated interacting protein, the second is B subunit if known, the third is dephosphorylation site if known, the fourth is interacting boundary if known and the last row indicates the reference to the work documenting the interaction. Abbreviations were used based on Biogrid entries using Uniprot naming rules. Historical/common names are given in parenthesis where standard abbreviations are not commonly used or not easily interpretable. All proteins mentioned are human, with the exception of important human viral proteins, which have virus names indicated in [brackets].