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Role of Type 2C Protein Phosphatases in Growth Regulation and in Cellular Stress Signaling

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ABSTRACT A number of interesting features, phenotypes, and potential clinical applications have recently been ascribed to the type 2C family of protein phosphatases. Thus far, 16 different PP2C genes have been identified in the human genome, encoding (by means of alternative splicing) for at least 22 different isozymes. Virtually ever since their discovery, type 2C phosphatases have been predominantly linked to cell growth and to cellular stress signaling. Here, we provide an overview of the involvement of type 2C phosphatases in these two processes, and we show that four of them (PP2Cα, PP2Cβ, ILKAP, and PHLPP) can be expected to function as tumor suppressor proteins, and one as an oncoprotein (PP2Cδ/Wip1). In addition, we demonstrate that in virtually all cases in which they have been linked to the stress response, PP2Cs act as inhibitors of cellular stress signaling. Based on the vast amount of experimental evidence obtained thus far, it therefore seems justified to conclude that type 2C protein phosphatases are important physiological regulators of cell growth and of cellular stress signaling.

KEYWORDS signal transduction, cell growth, stress response, protein phosphorylation, serine/threonine-specific phosphatases, PP2C

1. INTRODUCTION

In the past few decades, substantial progress has been made in understanding the principles of cellular signal transduction. Through profound genetic and proteomic analyses, through the development of novel analytical techniques, and through the ever increasing ability to pharmacologically modulate cellular signal transduction, a significant number of signaling pathways have been identified, most of which have been shown to be highly conserved throughout phylogeny. In addition, most of these signaling pathways have been shown to be highly complex, both in their nature and in their regulation. As a result, only a small defect in one of the genes or proteins involved in the regulation of a single signaling pathway is often sufficient to allow disease development. In order to diagnose diseases as early as possible, as well as to develop optimized (or even personalized) treatment strategies, it is of the utmost importance to profoundly characterize both the mechanisms and the mediators of cellular signal transduction.

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Protein phosphorylation is arguably the most important mechanism for controlling cellular signal transduction (Hunter, 2000). In the process of protein phosphorylation, protein kinases are responsible for adding one or more phosphate groups to certain amino acid residues in their target proteins, while protein phosphatases are in charge of (subsequently) removing these phosphate groups. Protein kinases, of which approximately 500 different subtypes have been identified in the human genome (Manning *et al.*, 2002; Kostich *et al.*, 2005), have long been thought to be highly specific enzymes, recognizing and phosphorylating only a relatively small number of substrates. In contrast, protein phosphatases, of which only about 100 different subtypes have been identified thus far (Plowman *et al.*, 1999; Venter *et al.*, 2001), have generally been considered to be much more flexible enzymes, having a larger number of substrates and presenting with overlapping activities.

In recent years, this specificity issue has been questioned several times (Zhang *et al.*, 1993; Zhang, 1998, 2002; Tonks and Neel, 2001; Zhou *et al.*, 2002; Meskiene *et al.*, 2003). Due to the initial assumption, however, that kinases are more specific enzymes than phosphatases, and that they are therefore more interesting targets for developing specific therapeutic interventions, much more attention has been given to characterizing protein kinases. Only recently; fuelled largely by the realization that kinases are not as specific as initially thought (Zhou *et al.*, 2002; Meskiene *et al.*, 2003), as well as by the observation that targeting several different signaling pathways at once is not necessarily less effective or more toxic than targeting a single pathway (Bergers *et al.*, 2003; Erber *et al.*, 2004; McCarty, 2004; McCarty and Block, 2005), ever more effort is being put into the extensive evaluation of the function and clinical potential of protein phosphatases (Lyon *et al.*, 2002; van Huijsduijnen *et al.*, 2002; McClusky *et al.*, 2002; Schmid and Wocholski, 2004).

2. PROTEIN PHOSPHATASES

Protein phosphatases are structurally and functionally diverse enzymes that can be divided into three major families, based on their substrate specificity and on the conservation of their catalytic domains (Barford *et al.*, 1998). The first major family of phosphatases, the protein tyrosine phosphatases (PTPs), specifically dephosphorylate phosphotyrosine residues. They do, however, contain an additional subfamily of enzymes, the so called dual-specificity phosphatases (DSPs), that are also able to dephosphorylate phosphoserine and phosphothreonine residues. The second class of phosphatases, the phosphoprotein phosphatases (PPPs), as well as the third class, the metal-dependent protein phophatases (PPMs), are only able to dephosphorylate phosphoserine and phosphothreonine residues. The members of the PPP family can be further subdivided into three distinct subfamilies (PP1, PP2A, and PP2B) and are known to function as multimeric complexes. The PPM family, on the other hand, only contains a single subfamily (PP2C), and its members are known to function as monomers. In addition, as opposed to PPPs, PPMs are known to depend on bivalent cations (Mg^{2+} or Mn^{2+}) for their catalytic activity and to be insensitive to the broad-spectrum phosphatase-inhibitor okadaic acid.

3. THE TYPE 2C FAMILY OF PROTEIN PHOSPHATASES

Because of the increasing interest in targeting protein phosphatases for therapeutic purposes, we have here set out to provide an overview of the type 2C family of protein phosphatases, several members of which have been repetitively shown to be involved in the regulation of cell growth and cellular stress signaling. In recent years, the number of PP2C family members has grown steadily, and ever more interesting features, phenotypes, and potential clinical applications have been ascribed to this family of phosphatases. Thus far, at least 16 different PP2C genes have been identified in the human genome, encoding (by means of alternative splicing) for at least 22 different PP2C isozymes (see Table 1). Orthologs of human PP2Cs can be found in virtually all organisms, ranging from plants, bacteria, and yeast, to nematodes, insects, and mammals (Schweighofer *et al.*, 2004). Such an extensive conservation throughout phylogeny indicates that these enzymes likely play important roles in regulating key cellular signaling events. Almost since their discovery, type 2C phosphatases have been predominantly linked to cell growth and to cellular stress signaling. Before focusing on the involvement of each individual PP2C family member in these two processes in mammals, we will address the regulation of the phosphatases themselves, as well as their role in regulating cell growth and cellular stress signaling in lower organisms, like plants and yeast.

TABLE 1 **Overview over the human type 2C protein phosphatases identified thus far**

Name	Aliases	Gene Locus	Isoforms	Protein Size (Primary Isoform)	Cellular Localization
PP2Cα	PPM1A	14q23.1	1:NP_066283 2: NP_808820 3: NP_808821*	42 kDa	Cytoplasm Nucleus
PP2Cβ	PPM1B	2p22.1	1: NP_002697 2: NP_808907 3: NP_808908 4: NP_001033556 5: NP_001028729	53 kDa	Cytoplasm
PP2Cγ	PPM1G	2p23.3	1: NP_817092 2: NP_002698*	59 kDa	Nucleus
PP2Cδ**	PPM1D, WIP1	17q23.3	1:NP_003611	67 kDa	Nucleus
PP2Cε	PPM1L	3q25.33-q26.1	1: NP_640338	20 kDa	ND
PP2Cζ	PPM1J	1p13.2	1: NP_005156	33 kDa	ND
PP2Cη	PPM1M	3p21.2	1:NP_653242	30 kDa	Nucleus
PP2Cκ	PPM1K	4q22.1	1: NP_689755	41 kDa	Nucleus
CaMKP	PPM1F, POPX2, hFEM-2	22q11.22	1: NP_055449	50 kDa	Cytoplasm
CaMKP-N	PPM1E, POPX1	17q23.2	1: NP_055721	84 kDa	Nucleus
ILKAP	PP2Cδ**	2q37.3	1: NP_110395 2: NP_789769	43 kDa	Cytoplasm
PHLPP	SCOP PLEKHE1	18q21.33	1: NP_919431	134 kDa	Cytoplasm Nucleus Mitochondrion
NERRP-2C	PPM1H ARHCL1	12q14.1-q14.2	1: XP_350881	47 kDa	ND
TA-PP2C	PPTC7	12q24.11	1: NP_644812	33 kDa	ND
PDP1	PPM2C	8q22.1	1: NP_060914	61 kDa	Mitochondrion
PDP2	—	16q22.1	1: NP_065837	60 kDa	Mitochondrion

*Indicates that the respective isoform only differs from the primary isoform in its 5′-UTR and that it thus encodes for the same protein as the primary isoform. **Indicates that ILKAP has recently also been referred to as PP2Cδ. ND: Not (yet) determined.

3.1. Regulation of Type 2C Protein Phosphatases

Unlike their PPP counterparts, which function as homo- and hetero-, di- and trimeric complexes, PP2Cs are monomeric enzymes. Therefore, they are not being regulated by inhibitory proteins or by regulatory subunits. Because of the fact that the intracellular concentrations of Mg^{2+} and Mn^{2+}do not fluctuate substantially under physiological conditions, it is also unlikely that these metal-dependent phosphatases are regulated by the bivalent cations that they require for their catalytic activity. Based on these two observations, it is expected that the activities of type 2C protein phosphatases are controlled predominantly by 1) their tissue- or cell type-specific expression, by 2) their post-translational modification, by 3) their subcellular compartmentalization, and 4) by their degradation.

3.2. Functions of Type 2C Protein Phosphatases in Plants

In Arabidopsis, more than 70 PP2C genes have been identified thus far (Kerk *et al.*, 2002). In a recent review, Schweighofer and colleagues (2002) clustered these genes into ten different groups, based on their sequence similarity. Two of these groups could be linked specifically to growth regulation and to the cellular stress response; group A, which includes ABI1 and ABI2, and which is associated to abscisic acid (ABA) signaling, and group B, which is characterized by its homology to MP2C, an alfalfa type 2C phosphatase that is known to be involved in mitogen-activated protein kinase (MAPK) signaling. As for the former, several lines of evidence have been provided, showing that plant PP2Cs act as negative regulators of the ABA signaling pathway, one of the most important growth and stress signaling pathways in plants (Federoff, 2002; Himmelbach *et al.*,

2003). Additional experiments showed that this down-regulation was specific for type 2C phosphatases, as mutations in other phosphatases (like PP1, PP2A, or PP2B) did not affect ABA-mediated signal transduction (Gosti *et al.*, 1999). Concerning the latter group of plant PP2Cs, a yeast two-hybrid screen has demonstrated that MP2C interacts directly with SIMK (stress-induced MAPK) (Bogre *et al.*, 1997; Meskiene *et al.*, 1998, 2003). Upon wounding-induced stress, the expression of MP2C was found strongly to be induced in the leaves of the plants, in a manner that correlated well with the timing of SIMK inactivation. These observations indicate that, even in lower organisms like Arabidopsis, type 2C protein phosphatases are involved in (down-)regulating cell growth and cellular stress signaling.

3.3. Functions of Type 2C Protein Phosphatases in Yeast

In yeast, seven PP2C genes have been identified thus far (Jiang *et al.*, 2002). As in plants, the yeast PP2Cs, *i.e.*, the PTCs (phosphatase two C's), have been shown to be involved consistently in regulating cell growth and cellular stress signaling. The first report addressing the function of PTCs in yeast showed that Ptc1 is important for survival upon heat-shock-induced stress (Shiozaki *et al.*, 1994). In a subsequent report, the same authors also showed that Ptc1 plays a key role in osmoregulation, that it is upregulated in response to osmotic stress, and that it counteracts the function of MAPKK WIS1 (Shiozaki and Russell, 1995). Several years later, two additional type 2C phosphatases, Ptc2 and Ptc3, which differ from Ptc1 in a having an additional noncatalytic domain, were also shown to be involved in the regulation of the yeast MAPK (HOG1) pathway. Elegantly designed experiments have shown that, whereas Ptc1 controls the basal levels of HOG1, as well as its dephosphorylation and inactivation during adaptation, Ptc2 and Ptc3 limit the maximal activation of the HOG1 pathway (Young *et al.*, 2002). Furthermore, Ptc2 has been implicated in the unfolded protein response (Welihinda *et al.*, 1998) and in checkpoint signaling (Marsolier *et al.*, 2000), and both Ptc2 and Ptc3 have been shown to be required for the adaptation and the recovery after endonuclease-induced double-strand breaks (Leroy *et al.*, 2003).

In addition to their role in regulating cellular stress signaling, Ptc2 and Ptc3 have also been shown to be involved in growth regulation. In eukaryotic cells, cell growth and cell cycle progression are controlled predominantly by the sequential activation and inactivation of cyclin-dependent protein kinases (CDKs), the activities of which are regulated to a large extent by protein (de-)phosphorylation (Morgan, 1995; Solomon and Kaldis, 1998). Evidence for a role of type 2C phosphatases in coordinating cell growth in yeast has been provided by Solomon and colleagues (Cheng *et al.*, 1999), who showed that Ptc2 and Ptc3 are responsible for removing the activating phosphorylation of Cdc28. As Cdc28 is generally considered to be the primary physiological regulator of cell cycle progression in yeast, these findings indicate that, as in plants, type 2C phosphatases are not only important for controlling cellular stress signaling, but also for regulating cell growth.

4. FUNCTIONS OF TYPE 2C PROTEIN PHOSPHATASES IN MAMMALS

In humans, 16 different PP2C family members have been identified thus far. By means of alternative splicing, these 16 genes encode for at least 22 different PP2C isozymes (see Table 1). Homologs displaying a high degree of sequence similarity to human PP2Cs have also been identified in mice, rats, and cows. In the following paragraphs, we provide a concise overview over the involvement of each individual (human) PP2C family member in cell growth and in cellular stress signaling.

4.1. Protein Phosphatase 2Cα (PPM1A)

PP2Cα is the best characterized member of the type 2C family of protein phosphatases. It was first identified in 1992, using a rat liver library and a human teratocarcinoma library (Mann *et al.*, 1992). A few years later, the molecular architecture of human PP2Cα was determined (Das *et al.*, 1996). It revealed a novel protein fold that consists of two domains; an N-terminal catalytic domain, that is composed of a central β-sandwich surrounded by α-helices and that is common to all PP2Cs, and a 90-residue C-terminal domain, that merely contains α-helices and that is characteristic for mammalian PP2Cs. This latter domain is remote from the catalytic site, suggesting that it has a role in defining substrate specificity. PP2Cα is expressed in virtually all tissues and is located in the cellular cytoplasm as well as the nucleus (Lifschitz-Mercer *et al.*, 2001). As an enzyme with broad substrate specificity, PP2Cα participates in the regulation of several important signaling pathways.

AMPK

The first piece of evidence for an involvement of PP2Cα in regulating cellular stress signaling has been provided by Hardie and colleagues (1995), who showed that PP2Cα dephosphorylates and inactivates AMP-activated protein kinase (AMPK) (Davies *et al.*, 1995). AMPK is the central component of a protein kinase cascade that is activated upon various different types of stress, especially upon those associated with ATP depletion, such as hypoxia, heat shock, and metabolic poisoning (Hardie, 1999). AMPK furthermore phosphorylates and inactivates NADPH, it mediates the responses of fatty acid and sterol synthesis pathways upon stress, and it is known to be important for assuring heart tissue homeostasis (Young *et al.*, 2005).

MAPK (JNK/p38)

As its plant and yeast homologs, PP2Cα has been shown to be involved in the regulation of mitogen-activated protein kinase (MAPK) signaling. MAPK cascades are central intracellular signaling modules that are composed of three tiers of sequentially activated proteins kinases: MAPKKKs, MAPKKs, and MAPKs. In mammalian cells, three distinct MAPK cascades can be found. The prototypic MAP kinases ERK1 and ERK2 are being controlled by mitogenic signaling through the MAPKKKs A-RAF, B-RAF, and C-RAF, and the MAPKKs MEK1 and MEK2 (Cobb and Goldsmith *et al.*, 1995). The two other MAPKs, *i.e.*, JNK and p38, are activated by stress, *e.g.*, by UV radiation, by heat shock, by osmotic shock, and by wound stress (Waskiewicz and Cooper, 1995). Upon such environmental stresses, PP2Cα has been shown to inhibit the activation of both the JNK and the p38 pathway (see Figure 1). As for the former, a downregulation of its signaling activity has been attributed to an inhibition at the level of MAPKKs; under physiological as well as under stress conditions, both MKK4 and MKK7, two major upstream regulators of JNK, were found to be dephosphorylated by PP2Cα (Hanada *et al.*, 1998). The regulation of the latter pathway, *i.e.*, the p38 pathway, by PP2Cα was also shown to be at the level of MAPKKs, as the overexpression of PP2Cα markedly suppressed the stress-induced phosphorylation of MKK3b and MKK6b, the two primary upstream activators of p38 (Hanada *et al.*, 1998). Furthermore, PP2Cα and p38 could be coimmunoprecipitated, indicating that they also interact directly (Takekawa *et al.*, 1998). Interestingly, however, they could only be coimmunoprecipitated when the cells were stimulated with stress, suggesting that PP2Cα only interacts with p38 when the latter is present in its phosphorylated (*i.e.*, stress-activated) form. As opposed to its involvement in controlling the activity of the two stress-activated MAPK pathways JNK and p38, PP2Cα did not affect the activity of the mitogen-activated ERK pathway (Zhou *et al.*,

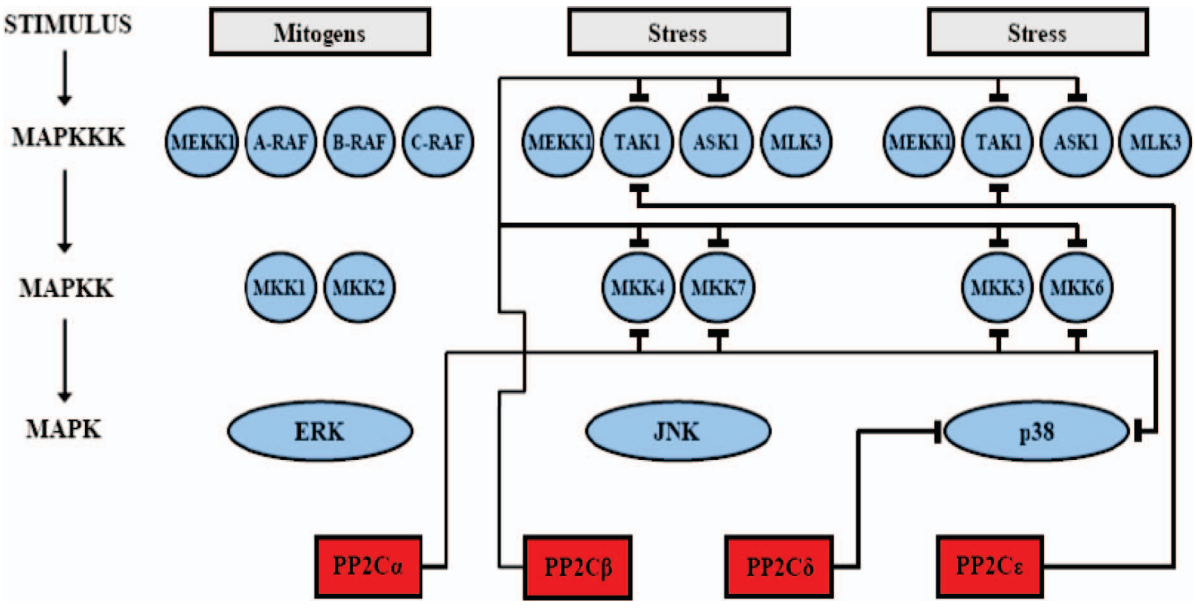

FIGURE 1 **Schematic representation of the involvement of type 2C protein phosphatases in regulating MAPK signaling. ⊥ Indicates inhibition of the respective protein, ↓ indicates activation.**

2002; Hanada *et al.*, 1998). This observation indicates that PP2Cα selectively inhibits cellular stress signaling.

CDK

Cell growth and cell cycle progression are being controlled predominantly by the sequential activation and inactivation of cyclin-dependent protein kinases (CDKs) (Morgan, 1995). To coordinate the cell cycle machinery, extra- and intracellular signals regulate the activities of CDKs. Besides through the association with regulatory subunits, through subcellular compartmentalization, through transcriptional control and through proteolysis, the activities of CDKs are being regulated to an important extent by the removal of inhibitory phosphorylations and by the addition of an activating phosphorylation (Solomon and Kaldis, 1998). As mentioned before, the primary regulator of cell cycle progression in yeast, Cdc28, has been shown to be dephosphorylated and inactivated by the yeast PP2Cs Ptc2 and Ptc3 (Cheng *et al.*, 1999). In mammalian cells, cell cycle progression is not controlled by one, but by several CDKs, *e.g.*, by Cdk1 (Cdc2), Cdk2, Cdk4, and Cdk6. In their report describing the impact of Ptc2 and Ptc3 expression on the phosphorylation status of Cdc28, Cheng and colleagues (2000) also showed that, in human (HeLa) cells, PP2C-like activities were responsible for >99% of the phosphatase activity acting on Cdk2. This final points toward an evolutionary conserved mechanism for type 2C protein phosphatases in controlling cell cycle progression. In a subsequent report, the same authors further characterized this interaction, showing that both PP2Cα and PP2Cβ2 were able to dephosphorylate both Cdk2 and Cdk6 (Cheng *et al.*, 2000). These two studies were the first to demonstrate that PP2Cα plays an important role in regulating cell cycle progression and cell growth.

p53/MDM2

Additional evidence for an involvement of PP2Cα in controlling cell cycle regulation, cell growth, and cellular stress signaling has been provided by Lavi and colleagues (Ofek *et al.*, 2003). Upon the tetracycline-induced overexpression of PP2Cα, they found that HEK293 cells failed to proliferate and to form colonies. In addition, they observed that the overexpression of PP2Cα resulted in cell cycle arrest in G2/M and in apoptosis. When investigating this phenomenon more closely, it was found that PP2Cα was able to induce both the expression and the transcriptional activity of p53, the so called 'guardian of the genome' (Lane, 1992), and undoubtedly one of the most important regulators of cell growth and cellular stress signaling (Levine, 1997; Vogelstein *et al.*, 2000). To confirm the validity of this finding, Lavi and colleagues (2003) then went on to show that also in HCT116 cells, PP2Cα increases the transcriptional activity of p53, and that it does so in a p53 dose-dependent manner (Ofek *et al.*, 2003). Furthermore, using cells that stably express the human papilloma virus E6 protein, which is known to induce p53 ubiquitination and degradation, it could be demonstrated that the inhibition of colony formation induced by the overexpression of PP2Cα was mediated (at least in part) by p53. These findings indicated, for the first time, that PP2Cα is an important physiological regulator of p53 signaling (see Figure 2).

In a subsequent report, the same authors then extended their efforts by showing that PP2Cα mediates these effects by downregulating the expression and the activity of MDM2, the primary endogenous inhibitor of p53 (Ofek *et al.*, 2007). They first confirmed that the overexpression of PP2Cα stabilizes and activates p53, both in primary cells and in mouse embryonic fibroblast cells (MEFs) in which p53 was first knocked out and than reintroduced. This latter finding, as well as the results obtained through experiments using PP2Cα siRNA-expressing cells and the proteasome inhibitor ALLN, indicated that PP2Cα conferred these effects by reducing the ubiquitin-dependent degradation of p53. As MDM2 is known to be the primary E3 ligase responsible for ubiquitinating p53 (Michael and Oren *et al.*, 2003), the authors next assessed the impact of both the overexpression and the downregulation of PP2Cα on the expression of MDM2. As hypothesized, upon the siRNA-mediated knockdown of PP2Cα, they indeed detected significantly increased levels of MDM2 in cells treated with UV irradiation or doxorubicin (Ofek *et al.*, 2007). Conversely, by increasing the expression of PP2Cα, the levels of MDM2 could be reduced, an effect which turned out to be specific, as a phosphatase-deficient mutant of PP2Cα failed to downregulate MDM2. In addition, Ofek and coworkers (2007) provided evidence showing that these effects were independent of p53, as PP2Cα also controlled the levels of MDM2 in cells lacking p53 or in cells expressing mutant p53. By finally showing that PP2Cα exerted these effects predominantly by increasing the proteasomal degradation of MDM2 through 'stress-induced MDM2 autoubiquitination' (SAMA), they have provided a

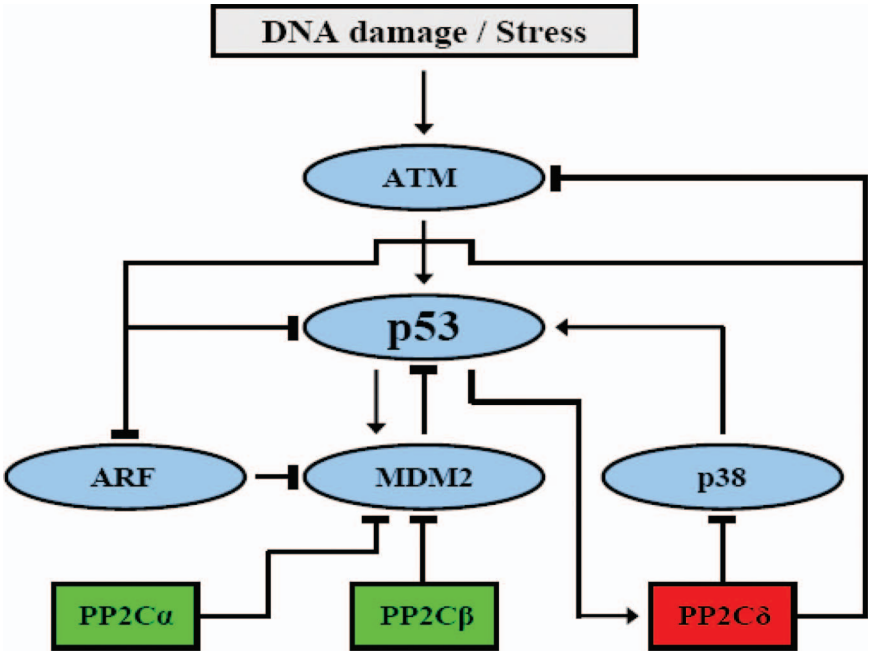

FIGURE 2 **Schematic representation of the involvement of type 2C protein phosphatases in regulating p53 signaling. ⊥ Indicates inhibition of the respective protein, ↓ indicates activation.**

rational explanation for the observation that PP2Cα upregulates p53 (Ofek *et al.*, 2007). These findings clearly classify PP2Cα as an important physiological regulator of the p53-MDM2 feedback loop.

TGFβ/Smad

Transforming growth factor beta (TGFβ) signaling regulates a variety of different processes in a variety of different cells (Akhurst and Derynck, 2001; Bierie and Moses, 2006). In contrast to the diverse and complex set of cellular responses that are regulated by TGFβ (*e.g.*, proliferation, differentiation, matrix remodeling, migration, and apoptosis), the signaling cascade downstream of TGFβ is relatively straightforward and simple; first, TGFβ binds to the type II TGFβ-receptor (TβRII) on the cellular membrane, resulting in the phosphorylation and the activation of the type I TGFβ-receptor (TβRI) (Akhurst and Derynck, 2001). TβRI then phosphorylates the receptor-activated Smads (R-Smads) Smad2 and Smad3 on two serine residues in their C-terminal SXS motif, thereby altering their conformation and thereby inducing their release from the hetero-oligomeric receptor complex. The released R-Smads subsequently form a complex with the common Smad (Co-Smad) Smad4 and they translocate to the nucleus, where they interact with different types of transcription factors to regulate gene expression in a context-dependent manner (Feng and Derynck, 2005).

For quite a while, the potential of R-Smad dephosphorylation as a regulatory mechanism for inhibiting TGFβ signaling and the phosphatase responsible for this dephosphorylation have remained elusive. Recently, however, through a functional genomic approach, Feng and colleagues (2006) have been able to show that PP2Cα is the long sought-after R-Smad phosphatase (Lin *et al.*, 2006). Upon having demonstrated that the dephosphorylation of Smad2 and Smad3 was mediated by an okadaic acid-insensitive phosphatase, they evaluated the abilities of 39 okadaic acid-insensitive phosphatases to dephosphorylate Smad2 and Smad3, and they found that only PP2Cα was able to do so. To confirm this finding, they subsequently showed that two phosphatase-dead mutants of PP2Cα failed to dephosphorylate Smad2 and Smad3, that PP2Cα dephosphorylated Smad2 in a cell-free assay, that the dephosphorylation of Smad2 and Smad3 was highly dependent on magnesium, and that the knockdown of PP2Cα resulted in increased levels of phosphorylated Smad2 and Smad3 (Lin *et al.*, 2006). In addition, they demonstrated that by dephosphorylating Smad2 and Smad3 in the nucleus, PP2Cα negatively affected the complex formation between Smad2 and Smad4 and between Smad3 and Smad4, thereby inducing the nuclear export of the two R-Smads. And furthermore, they showed that an induced expression of PP2Cα resulted in resistance to TGFβ and, conversely, that the

knockdown of PP2Cα sensitized cells toward TGFβ. Based on these findings, they propose that PP2Cα terminates TGFβ signaling by dephosphorylating the critical SXS motif of Smad2 and Smad3, by dissociating the complex between Smad2 and Smad4 and between Smad3 and Smad4, and by inducing the nuclear export of Smad2 and Smad3.

TGFβ is known to act both as a tumor suppressor and as a stimulator of tumor progression, invasion, and metastasis (Cui *et al.*, 1996). At the early stages of tumorigenesis, it acts directly on the cancer cells to suppress tumor outgrowth. At later stages, on the other hand, genetic and/or biochemical changes allow TGFβ to stimulate tumor progression by its pleiotropic effects on both the cancer cells themselves and the nonmalignant stromal cells that are present within solid tumors (Akhurst and Derynck, 2001; Bierie and Moses, 2006). Because of the fact that the majority of human tumors retain a functional TGFβ signaling pathway, it is generally assumed that the induction of invasion and metastasis by TGFβ is of greater clinical importance than is its role in tumor suppression (Akhurst and Derynck, 2001), which is exemplified by the establishment and the (pre-) clinical evaluation of several different TGFβ-inhibitors (Bierie and Moses, 2006). Taking the abovementioned observations into account, it can therefore be expected that the inhibition of TGFβ signaling contributes to the growth-inhibitory and tumor-suppressive properties of PP2Cα.

Wnt/Axin

Genes of the Wnt ('wingless type') family are differentially regulated during development. They encode for glycoproteins that are putatively involved in signal transduction, in the determination of cell fate and in oncogenesis (Nusse and Varmus, 1992). Using a yeast two-hybrid screen, Sussman and colleagues (2000) have shown that PP2Cα associates with a complex of Dishevelled, β-catenin and Axin, the latter being the primary endogenous inhibitor of Wnt signaling (Strovel *et al.*, 2000). By means of a Wnt-responsive LEF-1 reporter gene assay, they also demonstrated that, as opposed to the expression of several phosphatase-dead point mutants, the expression of functional PP2Cα activated LEF-1-mediated transcription. In addition, Strovel and colleagues (2000) have provided evidence for the mechanism by which PP2Cα induces the transcriptional activity of LEF-1, showing that by directly dephosphorylating Axin, and by thereby decreasing its half-life, PP2Cα relieves the Axin-mediated repression of LEF-1-dependent transcription. As Wnt signaling and LEF-1-mediated transcription are known to induce proliferation and malignant transformation (Nusse and Varmus, 1992; Smalley and Dale, 1999; Reya and Clevers, 2005), these findings indicate that in addition to acting as an inhibitor of cell growth, under certain conditions, PP2Cα is also able to promote cell growth. Because of the observations made in the experiments evaluating the direct effects of PP2Cα overexpression, however, PP2Cα should be considered a negative regulator of cellular proliferation (Kobayashi *et al.*, 1994; Ofek *et al.*, 2003).

4.2. Protein Phosphatase 2Cβ (PPM1B)

As PP2Cα, PP2Cβ was first identified using a rat liver library and a human teratocarcinoma library (Mann *et al.*, 1992). Investigation of the transcripts of the PP2Cβ gene revealed that there are at least five different PP2Cβ isoforms (Wenk *et al.*, 1992; Terasawa *et al.*, 1993; Hou *et al.*, 1994; Ito *et al.*, 1995). As these isoforms only differed in their C-terminal domains, it was suggested that this terminus is responsible for determining substrate specificity (Kusuda *et al.*, 1998). Expression analyses in mice revealed that the two primary isoforms, *i.e.*, PP2Cβ1 and PP2Cβ2, are expressed ubiquitously, whereas the isoforms PP2Cβ3, PP2Cβ4, and PP2Cβ5 are expressed exclusively in the intestine and in adult mouse testes (Wenk *et al.*, 1995; Ohnishi *et al.*, 1996). In the latter tissue, the expression of these three isoforms was found to increase significantly during the first wave of spermatogenesis, indicating that they are regulated in a differentiation-dependent manner (Ohnishi *et al.*, 1996). A later report confirmed that PP2Cβ1 and PP2Cβ2 are indeed expressed ubiquitously, and it also showed that their expression levels were highest in skeletal muscle and in heart (Seroussi *et al.*, 2001). In addition, as opposed to an earlier study, which had indicated that all PP2Cβ isoforms were present in the cytoplasm as well as in the nucleus (Wenk and Wieskes, 1995), Seroussi and colleagues (2001) observed that PP2Cβ1 and PP2Cβ2 were localized exclusively in the cytoplasm. In line with the report by Wenk and Wiekes, the Seroussi study indicated that overexpression of PP2Cβ leads to growth arrest and cell death, suggesting that as PP2Cα, PP2Cβ is an inhibitor of

cell growth. As described below, several subsequent reports have confirmed these initial observations, and they have also provided evidence showing that PP2Cβ is an important negative regulator of cellular stress signaling.

MAPK (p38/JNK)

Experiments performed by Tamura and coworkers (1998) have shown that PP2Cβ is actively involved in the regulation of the stress-activated MAPK pathways p38 and JNK (Hanada *et al.*, 1998). Initially, they observed that both under basal and under stress conditions, the overexpression of PP2Cβ1 reduced the levels of phosphorylated p38. Subsequently, they showed that this was primarily due to the dephosphorylation of upstream activators of p38, as the overexpression of PP2Cβ1 resulted in reductions in the levels of phosphorylated MKK3b and MKK6b. In addition, they provided evidence showing that the overexpression of PP2Cβ1 in COS7 cells reduced the phosphorylation status and the activity of MKK4 and MKK7, two important upstream regulators of JNK. As a consequence, reduced levels of phosphorylated JNK were detected in these cells. To confirm these findings, they performed several experiments using phosphatase-dead mutants of PP2Cβ1, and as hypothesized, increased phosphorylation levels were observed for all MKKs tested. In line with a report by Zhang and colleagues (Zhou *et al.*, 2002), Tamura and coworkers (1998) finally also observed that the overexpression of PP2Cβ1 neither affected the phosphorylation status and the activity of ERK1, nor those of its primary upstream regulator, MKK1 (Hanada *et al.*, 1998). These findings strengthen the conclusion that PP2Cβ selectively suppresses stress-activated MAPK pathways (see Figure 1).

TAK1

Additional evidence pointing towards an involvement of PP2Cβ in controlling the activities of JNK and p38 was provided by the same authors a few years later (Hanada *et al.*, 2001). Using coimmunoprecipitation analyses, they demonstrated that PP2Cβ1 associates with TAK1 (TGFβ activated kinase; MAPKKK7), an MKKK that is known to be activated by various different types of stress (Shirakabe *et al.*, 1997; Zhang *et al.*, 2000; Wang *et al.*, 2001), and that activates both the MKK4-JNK and the MKK6-p38 pathway (Yamaguchi *et al.*, 1995). Whereas the expression of intact PP2Cβ1 was shown to result in the dephosphorylation and the inactivation of TAK1, the expression of a phosphatase-dead mutant of PP2Cβ1 inhibited this dephosphorylation, and it increased TAK1 signaling (Hanada *et al.*, 2001). These findings demonstrate that also by dephosphorylating the MAPKKK TAK1, PP2Cβ attenuates the activities of the two stress-activated MAPK pathways JNK and p38.

IKKβ/NF-κB

The NF-κB pathway is an important physiological regulator of immune and inflammatory responses (Baeuerle and Baltimore, 1996; Baldwin, 1996), as well as of apoptosis (Karin and Lin, 2002; Shishodia and Aggarwal, 2002), and of the cellular stress response (Wang *et al.*, 2002; Li and Karin, 1999). One of the critical events in the activation of the NF-κB pathway is the stimulation of IκB kinases (IKKs) by cytokines like transforming growth factor-α (TGFα) and interleukin-1 (IL-1) (Karin and Ben-Neriah, 2000). Gaynour and colleagues (2004) have provided evidence showing that PP2Cβ associates with IKKβ, the primary endogenous activator of NF-κB (Prajapati *et al.*, 2004). In addition, they showed that PP2Cβ dephosphorylates IKKβ, that it reduces the kinase activity of IKKβ, and that as a consequence, it decreases the transcriptional activity of NF-κB. The binding of PP2Cβ to IKKβ appeared to be decreased at the early time points after TGFα treatment and it was found to be restored at later time points. Using PP2Cβ siRNA, the authors were able to confirm their assumption that PP2Cβ is predominantly responsible for attenuating the activity of IKKβ at the later time points after cytokine stimulation. These findings led them to suggest that PP2Cβ is not only involved in attenuating the basal activity of IKKβ, but also in downregulating its activity after an initial stimulation by cytokines (Prajapati *et al.*, 2004). Taking into account that NF-κB is known to enhance survival, to inhibit apoptosis (Karin and Lin, 2002; Shishodia and Aggarwal, 2002), and to positively affect cellular stress signaling (Wang *et al.*, 2002; Li and Karin, 1999), the above mentioned observations indicate that by attenuating the activity of NF-κB, PP2Cβ functions as an inhibitor of cell growth and of cellular stress signaling.

CDK

As PP2Cα, PP2Cβ, or more specifically, PP2Cβ2 has been shown to be able to dephosphorylate the cyclin-dependent kinases Cdk2 and Cdk6 (Cheng *et al.*, 2000).

Using recombinant PP2Cβ2, Cheng and colleagues have provided evidence showing that this type 2C phosphatase only dephosphorylates monomeric Cdk2 and Cdk6, and not their cyclin-bound counterparts, confirming the findings of previous studies with yeast PP2Cs and human Cdk2 in HeLa cell extracts (Cheng *et al.*, 1999). Consistent with its evolutionary conserved ability to control the inactivation of cyclin-dependent kinases, PP2Cβ is thus likely to be an important inhibitor of cell cycle progression.

p53/MDM2

In their report evaluating the effects of the downregulation of PP2Cα on the expression and the activity of p53 and MDM2, Lavi and colleagues (2007) also addressed the impact of attenuating the expression of PP2Cβ (Ofek *et al.*, 2007). By comparing the level of MDM2 in cells expressing only PP2Cα siRNA with the level of MDM2 in cells expressing siRNAs directed against both PP2Cα and PP2Cβ, they were able to show that PP2Cβ is also involved in downregulating MDM2 and, thus, in increasing the stability and the activity of p53; when both phosphatases were knocked down simultaneously, the expression levels of MDM2 were found to be much higher than when only the expression of PP2Cα was reduced. Based on this observation, Ofek and coworkers (2001) then went on to address the physiological role of PP2Cβ in regulating the degradation of MDM2. Hereto, the level of MDM2 in lymphoblastoids derived from a Bedouin patient harboring a homozygous deletion of 179311 base pairs in chromosome 2p21, which includes the PP2Cβ gene (and which causes cystinuria, neonatal seizures, hypotonia, severe somatic and developmental delay, facial dysmorphism, and a reduced activity of respiratory chain complexes (Parvari *et al.*, 2001, 2005), was compared with the level of MDM2 in a 'normal' Bedouin sibling. As hypothesized, in the patient with 2p21 deletion syndrome, the expression of MDM2 was found to be increased substantially. In addition, the 'stress-activated MDM2-autoubiquitination' (SAMA), which was induced by cycloheximide, turned out to be inhibited in lymphoblastoids obtained from this patient. And furthermore, besides reducing the overall amount of MDM2-autoubiquitination, the knockout of the PP2Cβ gene was also found to slow down the onset of MDM2-autoubiquitination (Ofek *et al.*, 2007). Taking these findings into account, it seems to justified to conclude that PP2Cβ plays an important role in the regulation of the p53-MDM2 feedback loop (see Figure 2).

BAD

By demonstrating that PP2Cβ is able to dephosphorylate and activate the 'Bcl-2/Bcl-xL-associated death promotor' BAD, Krieglstein and colleagues (2003) have provided evidence indicating that PP2Cβ is involved in the regulation of apoptosis (Klumpp *et al.*, 2003). BAD is an important pro-apoptotic member of the Bcl-2 family of proteins. By directly interacting wit Bcl-xL through its BH3 homology domains, BAD interferes with the anti-apoptotic activities of Bcl-xL (Zha *et al.*, 1997). The formation of the BAD-Bcl-xL-heterodimer is determined solely by the phosphorylation status of BAD (Lizcano *et al.*, 2000; Tan *et al.*, 2000). When phosphorylated, BAD is retained in the cytoplasm through its interaction with 14–3-3 proteins. Upon dephosphorylation, BAD is targeted to the mitochondrial membrane, where it binds to Bcl-xL, preventing it from exerting its anti-apoptotic activities. The phosphorylation status of Serine 155 in the BAD protein has been shown to be critical for its interaction with Bcl-xL (Lizcano *et al.*, 2000, Tan *et al.*, 2000). As Krieglstein and colleagues have demonstrated that PP2Cβ dephosphorylates exactly this residue in the BAD protein (Klumpp *et al.*, 2003), it can be expected that PP2Cβ plays a role in the regulation of mitochondrial apoptosis.

4.3. Protein Phosphatase 2Cγ (PPM1G)

PP2Cγ was initially identified as FIN13, *i.e.*, FGF-inducible gene 13, in an attempt to monitor the response of murine NIH-3T3 fibroblast cells to fibroblast growth factor 4 (FGF-4; K-FGF). In their initial report, Basilico and colleagues demonstrated that the FIN13 gene, which was found to be upregulated in response to FGF-4, encoded for a serine-threonine-specific phosphatase for which, at the time, neither a function nor a human homolog had been described (Guthridge *et al.*, 1996). In a subsequent report, the authors then went on to show that the FIN13 protein (*i.e.*, murine PP2Cγ) localizes exclusively to the nucleus and that its expression is restricted to embryos, to testes and to several other mouse tissues undergoing active proliferation, like the pregnant uterus, the placenta and stimulated ovaries (Guthridge *et al.*, 1997). They also showed that the

FIN13 protein depended on Mn^{2+} for its phosphatase activity and that it was insensitive to okadaic acid-mediated inhibition, confirming its classification as a type 2C protein phosphatase. Furthermore, they found that the overexpression of FIN13 suppressed colony formation in several different cell types, that it inhibited DNA synthesis, and that it resulted in an accumulation of cells in the G1 and the early S phase of the cell cycle (Guthridge *et al.*, 1997). These findings suggest that one of the physiological functions of (murine) PP2Cγ is to inhibit cell cycle progression and cell growth.

Not long after Guthridge and colleagues (1996) had cloned the murine PP2Cγ, Travis and Welsh (1997) identified the human PP2Cγ gene. It was found to encode for a type 2C phosphatase with a unique acidic domain, that, as its mouse homolog and its human family members, depended on Mn^{2+} and Mg^{2+} for its phosphatase activity. Tissue distribution studies showed that in humans, PP2Cγ is expressed ubiquitously, with the highest levels of transcripts being present in the testes, in the heart and in skeletal muscle. Thus far, except for the abovementioned results obtained for murine PP2Cγ (*i.e.*, FIN13), and for a study showing that human PP2Cγ is important for spliceosome formation and pre-mRNA splicing (Murray, 1995), no physiological functions have been attributed to PP2Cγ.

4.4. Protein Phosphatase 2Cδ (PPM1D/Wip1)

PP2Cδ was initially identified by Appella and colleagues (1991) as Wip1, *i.e.*, wild- type p53-induced phosphatase 1, as its expression was found to be upregulated in a p53-dependent manner in response to ionizing radiation (Fiscella *et al.*, 1997). As its type 2C family members, Wip1 turned out be insensitive to okadaic acid-mediated inhibition. It was found to be localized exclusively to the nucleus, and ectopic expression of Wip1 resulted in a suppression of colony formation. Independent from this investigation, Tong and colleagues (1998) identified human PP2Cδ as a ubiquitously expressed protein that was upregulated in response to different types of stress, *e.g.*, upon UV radiation or upon ethanol incubation. Overexpression of PP2Cδ in HEK293 cells was found to block cell cycle progression, to induce cell cycle arrest in early S, to inhibit DNA synthesis, and to induce cell death. Because of these findings, and because of the fact that it was a p53-induced gene, PP2Cδ was initially assumed to be a protein with growth inhibitory functions. Later experiments, however, convincingly demonstrated that it possesses growth-promoting, rather than growth-suppressing properties, and that it contributes substantially to the development of several different types of malignancy. As outlined below, over the past few years, a significant amount of evidence has been obtained showing that PP2Cδ is an oncogene, and that it exerts its effects by a variety of downstream mechanisms,*e.g.*,by reducing the activities of p38, p53, and ATM, by interfering with cell cycle checkpoints, and by negatively affecting base-excision repair.

MAPK (p38)

The first report describing an involvement of PP2Cδ in regulating cell growth and cellular stress signaling was provided by Imai and colleagues (Takekawa *et al.*, 2000). Besides being activated by IR- or UV-induced stress, they observed that the expression of PP2Cδ was also upregulated in response to oxidative (H_2O_2) and ribotoxic stress (anisomycin). In addition, they found that PP2Cδ dephosphorylated and inactivated the MAPK p38. As a consequence, PP2Cδ attenuated the stress-induced p38-mediated phosphorylation of p53 on Serine 38 and Serine 46, resulting in a reduced transcriptional activity of p53 and in an inhibition of p53-mediated apoptosis (Takekawa *et al.*, 2000). Taking into account that p53 induces the expression of PP2Cδ, that PP2Cδ dephosphorlylates and inactivates p38, and that this reduced activity of p38 results in a reduced transcriptional activity of p53, these three proteins seem to exist in a negative feedback loop that is likely to be of substantial importance for regulating both cell growth and cellular stress signaling (see Figure 2).

p53

Besides merely inactivating p53 by means of a p38-dependent mechanism, PP2Cδ has also been shown to dephosphorylate p53 directly (Lu *et al.*, 2005). When a p53 Serine 15 phosphopeptide was incubated with purified PP2Cδ in an in vitro phosphatase assay, PP2Cδ presented a high level of dephopshorylating activity that was shown to be both magnesium-dependent and okadaic acid-independent. Next, Lu, and colleagues (2005) incubated full-length Serine 15-phosphorylated p53 with increasing amounts of PP2Cδ and as hypothesized, they were able to show that PP2Cδ dephosphorylates p53 on this residue. The phosphorylation of p53

on Serine 15 is carried out by the kinases ATM and ATR in response to IR and UV irradiation, and it has been shown to be important for the apoptotic activity of p53 (Sluss *et al.*, 2004), as well as for its stability, as it inhibits the interaction of p53 with MDM2 (Shieh *et al.*, 1997). By analyzing the effects of IR and UV irradiation on the protein level and the phosphorylation status of p53 in mouse embryonic fibroblasts (MEFs), the authors subsequently provided additional evidence for an involvement of PP2Cδ in regulating p53 signaling (Lu *et al.*, 2005); they showed that in PP2Cδ-knockout MEFs, 5 Gy of IR increased the Serine 15 phosphorylation of p53 substantially. In MEFS expressing PP2Cδ, on the other hand, the phosphorylation of p53 on this residue was found to be reduced dramatically, demonstrating that PP2Cδ is a putative physiological inhibitor of p53 signaling. Moreover, Lu and colleagues (2005) show that in control U2OS cells, UV radiation increased both the overall level of p53, as well as the level of Serine 15-phosphorylated p53. Transfection of the cells with wild type PP2Cδ again attenuated Serine 15 phosphorylation, and likely as a direct result, it also reduced the overall level of p53 (by promoting its interaction with MDM2). Transfection with mutant PP2Cδ, or coexpression of PP2Cδ siRNA did not induce these effects, indicating that they were specific for PP2Cδ (Lu *et al.*, 2005). Taken together, these observations convincingly demonstrate that PP2Cδ plays an important role in regulating the activity and the stability of p53.

ATM

Additional evidence for an involvement of PP2Cδ in regulating cell growth and cellular stress signaling has been provided by Bulavin and colleagues (Shreeram *et al.*, 2006), who demonstrated that PP2Cδ dephosphorylates and inactivates the ataxia-telangietasia muted kinase (ATM). They showed that in PP2Cδ-knockout cells, the ATM signaling cascade was activated, and that in PP2Cδ-overexpressing cells, the activity of the ATM pathway was attenuated. In addition, they demonstrated that PP2Cδ physically interacts with ATM even in unstressed cells, which seems to point towards a mechanism by which PP2Cδ assists in setting a threshold for the initial activation (*i.e.*, the phosphorylation) of ATM. The tumor suppressor protein ATM is known to be a master regulator of cell cycle checkpoints after DNA damage. It has long been thought to function specifically after IR-induced DNA double-strand breaks, but accumulating evidence suggests that it responds ubiquitously to several types of stress (Kastan and Bartek, 2004; Bakkenist and Kastan, 2004). ATM (co-)controls the activities of several different signaling pathways involved in cell cycle regulation and in the cellular stress response (*e.g.*, p53; see Figure 2), and its net effects include cell cycle arrest, activation of DNA repair and induction of apoptosis (Kastan and Lim, 2000). Taking the abovementioned observations into account, it seems reasonable to assume that by dephosphorylating and inactivating ATM, PP2Cδ functions as a positive regulator of cell growth and as an inhibitor of cellular stress signaling.

Chk1

By means of coimmunoprecipitation analyses, Lu and colleagues (2005) have shown that PP2Cδ interacts with checkpoint kinase 1 (Chk1), besides p53 and ATM, yet another important regulator of cell cycle progression and cellular stress signaling (Lu *et al.*, 2005). PP2Cδ was found to dephosphorylate Chk1 on Serine 345 and Serine 317, and as a result, the overexpression of PP2Cδ significantly reduced the kinase activity of Chk1 upon UV-induced stress. By subsequently showing that several breast cancer cell lines that endogenously express high amounts of PP2Cδ exhibited an attenuated UV-induced Serine 345 phosphorylation as compared to cells expressing only low amounts of the phosphatase, the authors confirmed that PP2Cδ plays a physiological role in regulating Chk1 signaling. Again using U2OS cells, they also demonstrated that PP2Cδ abrogates both the intra-S and the G2/M checkpoint upon IR and UV radiation. These findings led them to suggest that one of the primary functions of PP2Cδ is to reverse the p53- and the Chk1-induced cell cycle arrest, and to return the cells to a homeostatic state following the completion of DNA repair (Lu *et al.*, 2005).

Chk2

Not long after Lu and colleagues (2005) had demonstrated that PP2Cδ dephosphorylates Checkpoint kinase 1, Minami and coworkers showed that PP2Cδ also dephosphorylates Checkpoint kinase 2 (Chk2) (Fujimoto *et al.*, 2006). By means of a yeast two-hybrid screen and coimmunoprecipitation analyses, they first showed that Chk2 and PP2Cδ interact physically. Subsequently, upon inducing the phosphorylation of Chk2

by 10 Gy of IR, they observed that PP2Cδ dephosphorylated two Serine (S19 and S33/35) and two Threonine (T68 and T432) residues in Chk2. Because the expression of a phosphatase-deficient PP2Cδ mutant did not dephosphorylate these residues, these effects were considered to be specific. Furthermore, the authors showed that the siRNA-mediated knockdown of PP2Cδ resulted in an abnormally sustained Threonine 68 phosphorylation of Chk2, and that it increased the susceptibility of several cell types to IR. Several of the above mentioned findings were confirmed by Leteurtre and colleagues, who showed that in response to IR-induced DNA damage, PP2Cδ binds to Chk2 and dephosphorylates Threonine 68 (Oliva-Transtoy *et al.*, 2006). As a result, the overexpression of PP2Cδ suppressed the contribution of Chk2 to the IR-mediated induction of the G2/M checkpoint. Based on the results from these two studies, it seems to be justified to conclude that PP2Cδ is an important physiological inhibitor of Chk2 signaling in response to DNA damage.

UNG2/BER

Base excision repair (BER) is a central cellular mechanism for dealing with damaged DNA. Experiments performed by Lu and colleagues (2005) have provided evidence indicating that PP2Cδ is actively involved in the regulation of BER (Lu *et al.*, 2004). Overexpression of functional PP2Cδ was shown to suppress BER, while the expression of a point mutant lacking phosphatase activity enhanced BER. Using a yeast two-hybrid screen, they found that PP2Cδ physically interacted with UNG2, a nuclear uracil DNA glycosylase that is phosphorylated in a UV-dependent manner. They also observed that PP2Cδ dephosphorylated UNG2 at Threonine 6, one of the two sites that is phosphorylated in response to IR, and one of the sites that is important for initiating UNG2-mediated BER. As a result of this dephosphorylation, PP2Cδ inhibited several events associated with UNG2 function, like the initiation of uracil-mediated incisions and the final repair of uracil lesions. By means of truncation analyses, they furthermore obtained evidence suggesting that the binding of PP2Cδ to UNG2 may not merely be to facilitate catalytic dephosphorylation, but also, through its C-terminal domain, to recruit other factors to the complex, in order to maintain the suppression of BER (Lu *et al.*, 2004). Together with the abovementioned notions that PP2Cδ inactivates the BER-promotor p53 and the checkpoint kinases Chk1 and Chk2, these findings strengthen the conclusions that PP2Cδ plays a pivotal role in regulating the DNA damage response.

RSK2

P90 ribosomal S6 kinases (RSKs), of which four different isoforms have been identified thus far, are serine/threonine-specific kinases that have been implicated in a wide range of cellular functions, like transcriptional control (Xing *et al.*, 1996), cell survival (Shimamura *et al.*, 2000) and cellular proliferation (Frodin and Gammeltoft, 1999). Gammeltoft and coworkers (1999) have shown that RSK2, which is known to be activated by means of (ERK-mediated) phosphorylation, and PP2Cδ form a complex in vivo (Doehn *et al.*, 2004). Even though the authors were unable to demonstrate that PP2Cδ directly dephosphorylates and inactivates RSK2, the fact that the dephosphorylation of RSK2 in epidermal growth factor-stimulated cells was highly dependent on Mn^{2+} indicates that this was indeed the case. Furthermore, by means of coimmunoprecipitation analyses, Gammeltoft and colleagues (1990) showed that besides interacting with RSK2, PP2Cδ also associates with RSK1, RSK3, and RSK4, as well as with the 'mitogen- and stress-activated kinases' MSK1 and MSK2. These findings add several new proteins to the list of PP2Cδ substrates, and they suggest that by dephosphorylating and inactivating RSKs and MSKs, PP2Cδ can be expected to be involved in the control of transcription, of cellular survival and of cellular proliferation.

p16/p19

In addition to its role in regulating the activity of p53 and ATM, PP2Cδ has also been implicated in the control of two other tumor suppressor proteins; p16 (INK4A) and p19 (ARF) (Bulavin *et al.*, 2004; Bernards, 2004). The former is known to be an inhibitor of the cyclin D-CDK4-CDK6 protein kinases, which are upstream regulators of the retinoblastoma (Rb) tumor suppressor protein, and the latter is a well-known upstream regulator of p53 (Zhang *et al.*, 1998; Sherr, 2001; Sharpless, 2005). As opposed to all of the abovementioned target proteins of PP2Cδ, PP2Cδ does not interact directly with p16 and p19. Rather, upon evaluating the effects of PP2Cδ (Wip1) knockout in vitro and in vivo, Bulavin and colleagues (2004) demonstrated that PP2Cδ reduces the activities of p16 and p19 by

suppressing the activity of p38. First, they confirmed the results obtained by Imai and coworkers (Takekawa *et al.*, 2000), showing that the inactivation or the depletion of PP2Cδ results in an activation of p38. Next, they showed that the inactivation of PP2Cδ suppresses the oncogene-driven transformation of mouse embryonic fibroblasts (MEFs). Subsequently, they demonstrated that PP2Cδ-null MEFs not only express significantly increased amounts of p53, but also of p16 and p19, indicating that PP2Cδ simultaneously inactivates two different tumor suppressor pathways (*i.e.*, p53 and Rb). Using PP2Cδ- (Wip1-) knockout mice, they then extended their efforts by showing that deletion of PP2Cδ attenuates oncogene-driven mammary tumorigenesis *in vivo* (Bulavin *et al.*, 2004). Treatment with a specific inhibitor of p38 reconstituted the ability of the genetically modified mice to develop breast tumors, indicating that, in the absence of PP2Cδ, a constitutive activation of p38 prevents tumorigenesis. As these observations clearly classify PP2Cδ as a protein with oncogenic potential, in a subsequent report, Bulavin and colleagues then went on to show that chemical inhibitors of PP2Cδ may be useful anticancer agents (Belova *et al.*, 2005). *In vitro*, PP2Cδ inhibition decreased the proliferation rate of several breast cancer cell lines and it enhanced the growth inhibition induced by doxorubicin. *In vivo*, the PP2Cδ inhibitors were able to reduce the growth of xenograft tumors, as well as of tumors developed in MMTV-Neu transgenic mice. This led them to conclude that the pharmacological inhibition of PP2Cδ may prove to be an interesting approach for more effectively and more selectively treating certain types of solid malignancy (Belova *et al.*, 2005).

PP2Cδ is an Oncogene

Based on the abovementioned observations in mice, it can be expected that also in humans, PP2Cδ is involved in malignant transformation. Indeed, amplifications of 17q23, *i.e.*, the genomic region containing the PP2Cδ gene, have been repetitively observed in breast carcinoma specimens (Andersen *et al.*, 2002; Sinclair *et al.*, 2003), and they have been shown to correlate to a poor prognosis (Barlund *et al.*, 2000; Latham *et al.*, 2001). Several subsequent analyses have identified PP2Cδ as the primary cancer-causing gene in this region (Li *et al.*, 2002; Bulavin *et al.*, 2005; Rauta *et al.*, 2006), and they have furthermore demonstrated that besides being amplified in breast carcinomas, PP2Cδ is also overexpressed in ovarian clear cell adenocarcinomas (Hirasawa *et al.*, 2003), in neuroblastomas (Saito-Ohara *et al.*, 2003) and in medulloblastomas (Mendrzyk *et al.*, 2005). These notions clearly classify PP2Cδ as an oncogene.

4.5. Protein Phosphatase 2Cε (PPM1L)

The PP2Cε gene was first identified in the mouse genome, upon a homology search based on amino acid sequences that are typical for type 2C protein phosphatases (Li *et al.*, 2003). Shortly thereafter, the human PP2Cε gene was cloned and was found to be >99% identical to its mouse homolog (Jin *et al.*, 2004). As most of its family members, PP2Cε was found to be expressed ubiquitously.

TAK1/ASK1

Thus far, only for the mouse variant of PP2Cε, functional analyses have been performed. Tamura and colleagues have provided evidence showing that the overexpression of PP2Cε reduces the signaling activities of JNK and p38 in response to treatment with IL-1 (interleukin-1) or TAK1 (TGFβ-activated kinase 1; MAPKKK7) (Li *et al.*, 2003). Additional experiments showed that PP2Cε did not dephosphorylate p38 and JNK directly. Rather, it was found to act upstream of these two stress signaling molecules, by dephosphorylating the MAPKKK TAK1. Coimmunoprecipitation analyses demonstrated that the association of PP2Cε with TAK1 inhibited the binding of TAK1 to MKK4 and MKK6. Conversely, a dominant-negative variant of PP2Cε enhanced the interaction of TAK1 with MKK4 and MKK6, and it increased the overall activity of TAK1 in an AP-1 reporter gene assay. As Li and colleagues (2002) also noted that IL-1 treatment transiently suppressed the association between PP2Cε and TAK1, they propose a model in which, in the absence of IL-1 stimulation, PP2Cε contributes to keeping TAK1 signaling pathway in an inactive state.

A few years later, the same group of scientists showed that besides dephosphorylating TAK1, PP2Cε also dephosphorylates ASK1, another MAPKKK functioning upstream of JNK and p38. Through coexpression and coimmunoprecipitation analyses, they demonstrated that PP2Cε physically interacts with ASK1 (Tamura *et al.*, 2006). This finding could be confirmed in vivo, as also in mouse brain, PP2Cε was found to associate

with ASK1. In addition, it was shown that the (over-) expression of PP2Cε suppressed the H_2O_2-induced phosphorylation of ASK1 on Threonine 845, and that it inhibited the ASK1-mediated activation of AP-1, *i.e.*, the ultimate target the JNK pathway (Tamura *et al.*, 2006). Based on the results from these two studies, it seems to be justified to conclude that PP2Cε acts as a negative regulator of cellular stress signaling.

4.6. Protein Phosphatase 2Cζ (PPM1J)

As PP2Cε, PP2Cζ was first identified in the mouse genome (Kashiwaba *et al.*, 2003). As opposed to PP2Cε, however, PP2Cζ was not found to be expressed ubiquitously. Rather, it was found to be expressed specifically in the testes of adult mice. More detailed studies subsequently showed that PP2Cζ mRNA was exclusively produced in testicular germ cells, and that its expression increased during early neonatal development. Up to day 17 after birth, no mRNA could be detected in Northern blot analyses, at postnatal day 23, a faint band was observed, and from this day on, the intensity of the signal increased steadily. Therefore, Tamura and colleagues (2003) concluded that PP2Cζ is expressed specifically in testicular germ cells after the second stage of meiosis (Kashiwaba *et al.*, 2003).

UBC9

Using a yeast two-hybrid screen, the same group of scientists have also provided evidence showing that PP2Cζ physically interacts with UBC9 (ubiquitin-conjugating enzyme 9) (Kashiwaba *et al.*, 2003). UBC9 is an E2-conjugating enzyme that has been shown to associate with SUMO-1 (small ubiquitin-related modifier-1) during the course of sumoylation (Muller *et al.*, 2001). Through coimmunoprecipitation analyses, Kashiwaba and colleagues (2003) furthermore showed that the binding of SUMO-1 to UBC9 induces the recruitment of PP2Cζ to UBC9, or, alternatively, to a complex containing UBC9 and other sumoylation-related proteins. They suggest that in such complexes, PP2Cζ is responsible for the dephosphorylation of one (or more) of the constituents of the complex. As several important proteins, like p53, are known be sumoylated, and thus to potentially exist in complexes with SUMO-1, UBC9, and PP2Cζ, PP2Cζ can be expected to play a role in regulating the sumoylation and/or the phosphorylation status of these proteins.

4.7. Protein Phosphatase 2Cη (PPM1M)

As the two abovementioned type 2C protein phosphatases (PP2Cε and PP2Cζ), PP2Cη was identified by Tamura and coworkers (2003) in the mouse genome, again through a homology search based on amino acid sequences that are typical for PP2C family members (Komaki *et al.*, 2003). Besides cloning and analyzing the gene for PP2Cη, the authors also showed that as PP2Cζ, with whom it shares most sequence similarity, the highest transcript levels of PP2Cη could be found in testicular germ cells. As opposed to PP2Cζ, however, which was found to be expressed exclusively in testes, PP2Cη also turned out to be expressed in lung, in kidney, in brain, in heart and in liver. Tamura and colleagues furthermore showed that PP2Cη is located primarily in the nucleus (Komaki *et al.*, 2003). Thus far, no substrates of PP2Cη have been identified and no physiological functions have been attributed to this type 2C protein phosphatase.

4.8. Protein phosphatase 2Cκ (PPM1K)

The gene encoding for PP2Cκ was identified by Mao and coworkers (Dai *et al.*, 2006). They showed that PP2Cκ is a nuclear protein that contains a catalytic domain typical for type 2C phosphatases, which displays a high degree of sequence similarity to the catalytic domains of PP2Cα1 and PP2Cβ1. Tissue distribution experiments demonstrated that PP2Cκ is expressed ubiquitously, with the highest transcript levels being present in heart, brain, kidney, pancreas, and ovary. Dai and colleagues (2003) went on to show that the phosphatase activity of PP2Cκ was highly dependent on divalent cations (Mg^{2+} and Mn^{2+}), and that it was insensitive to okadaic acid-mediated inhibition, thereby confirming its classification as a type 2C phosphatase. In an attempt to identify signaling pathways that are being (co-)controlled by PP2Cκ, they also analyzed the transcriptional activity of several important cellular proteins (*e.g.*, p53, Rb, Myc, and NF-κB) in PP2Cκ-transfected cells and in cells transfected with empty vector. Out of the 10 transcription factors evaluated, only the 'heat shock response element' (HSE) was found to be activated, which points towards an involvement of PP2Cκ in regulating the (stress-activated) heat shock pathway (Dai *et al.*, 2006) Future experiments should aim to

confirm the validity of this finding, and to identify additional target proteins of PP2Cκ.

4.9. Calmodulin-Dependent Protein Kinase Phosphatase (CaMKP/PPM1F/POPX2/hFEM-2)

The gene encoding for the CaMKP protein was first cloned from a human myeloid cell line by Tabata and colleagues (Nomura *et al.*, 1994). Harvey and Ozer subsequently showed that this gene is highly similar to the rat CaMKP gene (Harvey and Ozer, 2001), which had been identified by Fujisawa and coworkers a few years earlier (Kitana *et al.*, 1999). CaMKP is expressed ubiquitously and it is localized exclusively in the cytoplasm.

CaMK

Both latter groups of authors went on to show that human CaMKP specifically dephosphorylates and inactivates CaMKII (Ishida *et al.*, 1998; Harvey *et al.*, 2004). Calmodulin-dependent protein kinases, like CaMKII, exhibit an extremely broad substrate specificity, they are abundantly expressed in the brain, and they are known to play an important role in the synthesis and release of neurotransmitters (Hudmon *et al.*, 2002), in long-term potentiation (Matynia *et al.*, 2002), in the regulation of carbohydrate metabolism, in ion channel function, in transcriptional control, in cytoskeletal organization, and in intracellular calcium homeostasis (Ishida *et al.*, 2003). Using coimmunoprecipitation analyses, Harvey and Ozer (2001) have provided evidence showing that CaMKP and CaMKII physically interact in vivo, and that the expression of CaMKP inhibits the phosphorylation of a CaMKII substrate. This led them to suggest that CaMKP is an important physiological regulator of CaMKII signaling (Harvey *et al.*, 2004).

PAK

At about the same time, using a yeast two-hybrid screen, Lim and colleagues (2002) identified the same protein as a type 2C protein phosphatase that associates with full length PIX, a guanine nucleotide exchange factor that interacts with PAK (p21-activated kinase; an upstream activator of the JNK pathway) (Koh *et al.*, 2002). They termed this protein POPX2, for partner of PIX 2. Pull-down experiments confirmed that a trimeric complex of these three proteins, *i.e.*, of PIX, PAK and POPX2, exists *in vivo*. The authors went on to show that PAK is a direct substrate of POPX2, and that POPX2 is able to dephosphorylate and inactivate PAK. Furthermore, upon microinjection of actived PAK into HeLa cells, they observed that the expression of POPX2 was able to block several of the phenotypic effects of PAK, like the breakdown of stress fibers and the morphological changes induced by $Cdc42^{V12}$, a putative upstream activator of PAK.

F1A

Also at about the same time, Yu and colleagues described the identification and the characterization of hFEM-2, a human homologue of the C. Elegans sex-determining protein FEM-2 (Tan *et al.*, 2001). They observed that the hFEM-2 protein displayed type 2C phosphatase activity and that it dephosphorylated CaMKII. In line with their finding that hFEM-2 is highly similar to rat CaMKP, hFEM-2 later turned out to be human CaMKP. In addition, Tan and coworkers (2001) showed that hFEM-2 associates with the Fas death domain-interacting protein F1Aα, and that its overexpression (as well as the overexpression of FEM-2 and of rat CaMKP) induces caspase-dependent apoptosis in several different cell lines. As only the intact hFEM-2 protein and a point mutant that retained its catalytic activity were able to promote apoptosis, and as two phosphatase-dead mutants failed to do so, it was concluded that hFEM-2 requires its phosphatase activity for promoting programmed cell death (Tan *et al.*, 2001). These findings suggest that CaMKP is involved in regulating cell growth by (co-)controlling apoptosis.

4.10. Nuclear Calmodulin-Dependent Protein Kinase Phosphatase (CaMKP-N/PPM1E/POPX1)

A few years after the identification of cytosolic CaMKP, Fujisawa and coworkers identified nuclear Calmodulin-dependent protein kinase phosphatase (CaMKP-N) (Takeuchi *et al.*, 2001). CaMKP-N was found to possess 64% sequence homology to CaMKP and to be expressed ubiquitously, with the highest transcript levels being present in the brain. In addition, it was found to be dependent on Mn^{2+} for its activity, confirming its classification as a type 2C protein phosphatase.

CaMK

Fujisawa and colleagues went on to show that CaMKP-N dephosphorylates the nuclear Calmodulin-dependent protein kinase CaMKIV, as well as nuclearly located CaMKII (Takeuchi *et al.*, 2001). In a subsequent report, the authors identified and characterized the nuclear localization sequences (NLS) of CaMKP-N, showing that there are two independent NLSs in the C-terminal portion of the protein. This finding is consistent with their previous conclusion that CaMKP-N exclusively dephosphorylates nuclear CaMKs (Takeuchi *et al.*, 2004).

PAK

As its closest relative (*i.e.*, CaMKP), CaMKP-N has been shown to physically interact with PIX and PAK (Tan *et al.*, 2001). Therefore, CaMKP-N is also known as POPX1, for partner of PIX 1. As opposed to CaMKP (POPX2), however, which is expressed at comparable levels in various tissues, CaMKP-N was found to be expressed predominantly in brain (Tan *et al.*, 2001). As the expression levels of PAK are also high in brain tissue, it can be assumed that POPX1 plays a more important role than POPX2 in dephosphorylating and inactivating PAK. Whether CaMKP-N, like CaMKP (*i.e.*, hFEM2), plays a role in regulating apoptosis, or whether it is involved in the regulation of other signaling pathways controlling cell growth and cellular stress signaling, has not yet been investigated.

4.11. Integrin-Linked Kinase-Associated Phosphatase (ILKAP)

The type 2C phosphatase ILKAP was identified by Hannigan and colleagues using a yeast two-hybrid screen and C-terminally truncated ILK1 (Integrin-linked kinase 1) as a bait (Leung-Hagesteijn *et al.*, 2001). ILKAP was found to be expressed ubiquitously and to be localized exclusively in the cytoplasm.

ILK/GSK3β

To confirm their direct interaction, ILK1 and ILKAP were coimmunoprecipitated from lysates of HEK293 cells. By inducing the expression of recombinant ILKAP in these cells, the kinase activity of ILK1, which was induced either by integrin activation or by growth factors, could be inhibited effectively. Conversely, a phosphatase-dead mutant of ILKAP failed to inhibit ILK1-mediated signal transduction, indicating that this effect was highly specific. Hannigan and colleagues have also provided evidence showing that ILKAP expression inhibited the integrin-induced phosphorylation of GSK3β, which is known to be a direct cellular target of ILK1 (Leung-Hagesteijn *et al.*, 2001). As a consequence, ILKAP attenuated the activity of the TCF/LEF family of transcription factors, which are the ultimate targets of the Wnt signaling pathway, and whose activation generally results in an increase in proliferation and in oncogenic transformation (Nusse and Varmus, 1992; Smalley and Dale, 1999; Reya and Clevers, 2005). In line with these results, in a subsequent report, the same group of scientists confirmed their initial findings, showing that the siRNA-mediated silencing of ILKAP stimulated the phosphorylation of GSK3β on Serine 9 (Kumar *et al.*, 2004). In the presence of ILKAP, on the other hand, the phosphorylation of GSK3β on this residue was found to be reduced, a 'phenotype' that could be rescued by the overexpression of functional ILK, but not by the overexpression of mutant ILK. Furthermore, they demonstrated that the expression of cyclin D1, a direct target of ILK-GSK3β signaling, was inversely correlated with ILKAP expression, suggesting that ILKAP is actively involved in cell cycle regulation. To confirm this assumption, they then went on to show in the absence of ILKAP-siRNA, the overexpression of ILKAP increased the number of cells in the G1 phase of the cell cycle. In the presence of ILKAP-siRNA, on the other hand, the amount of cells in G1 was found to be decreased and the number of cells entering the S phase was increased. As an additional means to confirm their findings, Hannigan and coworkers finally also showed that the overexpression of ILKAP reduced the anchorage-independent growth of LNCaP cells. Based on these findings, they conclude that ILKAP is an important physiological regulator of cell growth (Kumar *et al.*, 2004).

Together with the notion that deletions in 2q37.3, *i.e.*, the genomic region containing the ILKAP gene, have been causally linked to the development of oral carcinomas (Cengiz *et al.*, 2007), and with the fact that its direct substrate, *i.e.*, ILK1, has been shown to be overexpressed in a variety of human malignancies (Ewing's sarcoma (Chung *et al.*, 1998), prostate carcinoma (Graff *et al.*, 2001), melanoma (Dai *et al.*, 2003), and metastatic gastric carcinoma (Ito *et al.*, 2003)), the abovementioned observations strongly suggest that ILKAP is a potential tumor suppressor protein.

4.12. PH domain Leucine-Rich Repeat Protein Phosphatase (PHLPP/SCOP)

The gene for the type 2C phosphatase PH domain leucine-rich repeat protein phosphatase (PHLPP) was first identified by Nagai and colleagues as SCOP (for suprachiasmatic nucleus circadian oscillatory protein) (Shimizu *et al.*, 1999). They showed that PHLPP contains a PP2C-like domain, that it is expressed in most abundantly in the brain (but also in several other tissues), and that it is localized to the cytoplasm, the nucleus and the mitochondria (Shimizu *et al.*, 1999).

Akt

In order to identify a phosphatase involved in regulating Akt (PKB; protein kinase B) signaling, Newton and coworkers searched the available cDNA databases for a sequence that contains both a PH (pleckstrin homology) domain and a phosphatase domain (Gao *et al.*, 2005). The only sequence that they retrieved turned out to be abovementioned SCOP protein, of which the mRNA levels in the suprachiasmatic nuclei of rats were shown to be oscillating in a circadian rhythm-dependent manner (Shimizi *et al.*, 1999). Gao et al set out to further characterize this gene product and in line with the Shimizu study, they demonstrated that PHLPP is ubiquitously expressed, with the highest transcript levels being present in brain. In addition, they showed that PHLPP physically interacts with Akt, that it dephosphorylates the hydrophobic motif of Akt (*i.e.*, Serine 473), and that this dephosphorylation is insensitive to okadaic acid-mediated inhibition, thereby confirming the classification of PHLPP as a type 2C phosphatase. Using various different cancer cell lines, the authors furthermore showed that reduced levels of PHLPP correlate to increased levels of phosphorylated Akt, and that the transfection of these cells with PHLPP attenuates the amounts of phosphorylated Akt. Subsequently, they provided evidence indicating that by dephosphorylating Serine 473 in the Akt protein, PHLPP is able to induce apoptosis, a notion that was confirmed by the observation that the siRNA-mediated downregulation of PHLPP resulted in increased amounts of phosphorylated Akt and in an inhibition of apoptosis. And finally, Gao and colleagues (2005) also demonstrated that cancer cells transfected with PHLPP present with a substantially decreased growth rate *in vitro* and, even more importantly, with a significantly decreased tumorigenicity *in vivo*. Taking into account that Akt is known to be critical regulator of the balance between cell survival and apoptosis (Datta *et al.*, 1999), that the misregulation of Akt is considered to be a key cause of cancer (Vivanco and Sawyers, 2002), and that the loss of 18q21.33, *i.e.*, the genomic region containing the PHLPP gene, is frequently observed in human colon carcinomas (Jen *et al.*, 1994; Goal *et al.*, 2003), these findings suggest that by dephosphorylating and inactivating Akt, PHLPP may function as a tumor suppressor protein.

4.13. Additional Type 2C Family Members

In addition to twelve PP2C family members mentioned above, four additional proteins have been classified as type 2C protein phosphatases, either because they display a substantial degree of sequence similarity to known PP2Cs, or because possess characteristics typical for PP2Cs. As opposed to the twelve above mentioned PP2C isozymes, however, the following four type 2C phosphatases have either been only poorly characterized thus far, or they are biologically and functionally only distantly related to other PP2Cs.

NERPP-2C

The neurite extension-related protein phosphatase related to PP2C (NERRP-2C) was identified by Roach and colleagues, in an attempt to describe cDNAs that modify the growth of neurites on inhibitory myelin substrates (Lozano *et al.*, 1995). Additional analyses performed by the same group of scientists showed that (certain regions of) NERPP-2C displays substantial sequence similarity to type 2C protein phosphatases, that it depends on Mg^{2+} for its phosphatase activity and that its catalytic activity is okadaic acid-insensitive, thereby confirming its classification as a type 2C phosphatase (Labes *et al.*, 1998). Interestingly, however, as opposed to all other currently known PP2Cs, the authors also found that NERRP-2C was not only able to dephosphorylate phosphoserine substrates, but also phosphotyrosine substrates, indicating that it may also be a dual specificity phosphatase (DSP), and that it may thus belong to the PTP family of protein phosphatases. As it does not contain a sequence motif that is common to all other DSPs, however, and as it does contain 8 of 10 amino acid residues that are known to be essential for forming the phosphate- and metal-binding pocket in the catalytic center of type 2C phosphatases, they

termed NERRP-2C a 'dual specificity phosphatase, distantly related to PP2C.' In addition, they have provided evidence showing that NERRP-2C is expressed predominantly in the brain, that it plays a role in outgrowth of neurites and that it is involved in the regulation of neuronal signaling pathways responding to myelin-associated inhibitors (Labes *et al.*, 1998).

A few years later, upon characterizing the cDNA clones selected by GeneMark analysis from size-fractionated cDNA libraries from human brain, Ohara and colleagues identified the same gene as PPM1H (protein phosphatase magnesium-dependent 1H) (Hirosawa *et al.*, 1999). NERPP-2C/PPM1H is also known as ARHCL1, *i.e.*, the 'ras homolog gene family member C like 1' and its expected size is 47 kD. By analyzing GeneNote, a database of human genes and their expression profiles in healthy tissues that is based on array experiments performed at the Weizmann Institute of Science in Rehovot, Israel, it was found that the expression of NERPP-2C/PPM1H is not limited to the brain, but that it is expressed ubiquitously (*i.e.*,in all 12 tissues that were analyzed). Its cellular localization has not yet been investigated, and besides its involvement in the outgrowth of neurites and in the regulation of neuronal signaling pathways responding to myelin-associated inhibitors, no physiological functions have been attributed to NERPP-2C/PPM1H.

TA-PP2C

The T cell-activated protein phosphatase 2C (TA-PP2C) was identified by Linsley and colleagues, in a screen intended to identify genes that were upregulated in response to T cell-activation (Mao *et al.*, 2004). As hundreds of transcripts were found to be induced upon the activation of human peripheral T cells, the authors focused on those genes that were highly coregulated with the T cell-specific transcript interleukin-2 (IL-2). Among the genes that were highly coregulated with IL-2, besides the transcripts that were already known to function during T cell-activation, four genes represented previously unknown cDNA clones. One of these four genes turned out to encode for a type 2C protein phosphatase, as it possessed a domain characteristic for PP2C family members. Assuming that it is likely to play a role in T cell-activation, this gene was termed T cell-activated protein phosphatase 2C (Mao *et al.*, 2004). Thus far, no additional information has been obtained for TA-PP2C.

PDP1/PDP2

The pyruvate dehydrogenase phosphatases PDP1 and PDP2 are genetically and biologically distinct enzymes that consist of a catalytic and of a regulatory subunit (Huang *et al.*, 1998). The catalytic subunits of these two enzymes have been shown to be members of the type 2C family of protein phosphatases, as they were found to display a high degree of sequence similarity to rat PP2C and as they depended on Mg^{2+} for displaying their catalytic activity (Lawson *et al.*, 1993). Both proteins have been shown to be localized exclusively in the mitochondria (Huang *et al.*, 1998). PDP1 is predominantly expressed in the heart and in skeletal muscle, while PDP2 can be found in the liver, the kidney, the heart and in adipose tissue (Huang *et al.*, 1998, 2003). Thus far, both proteins have only been implicated in the regulation of the (activity of the) pyruvate dehydrogenase complex, which catalyzes the aerobic decarboxylation of pyruvate, and which thereby links glycolysis to the tricarboxylic acid cycle (Holness and Sugden, 2003).

5. CONCLUDING REMARKS

Collectively, the abovementioned observations demonstrate that type 2C protein phosphatases are broadly involved in regulating cell growth and cellular stress signaling. Table 2 summarizes the pathways and the proteins that are being (co-)controlled by PP2C-mediated dephosphorylation. The activity of several enzymes involved in MAPK signaling, for instance, has been shown to be regulated by type 2C phosphatases. Interestingly however, as depicted schematically in Figure 1, only those two MAPK tiers that have been linked to the cellular stress response (*i.e.*,p38 and JNK) are being controlled by PP2Cs. For the mitogen-activated MAPK pathway (*i.e.*,the ERK pathway), on the other hand, no evidence exists that its activity is being regulated by PP2Cs. In addition, several components of the p53 signaling pathway have been shown to be controlled by type 2C protein phosphatases (Figure 2). PP2Cα and PP2Cβ, for instance, have been shown to attenuate the expression and activity of MDM2, the primary physiological inhibitor of p53. As a consequence, the (over-)expression of these two phosphatases increases both the expression and the activity of p53. The (over-)expression of PP2Cδ, on the other hand, reduces both the expression and the activity of p53, and it has been shown to do so both by direct and by indirect mechanisms (Figure 2).

TABLE 2 **Overview over the involvement of type 2C protein phosphatases in cell growth and in cellular stress signaling.**

Type 2C phosphatase	Signaling pathway	Target protein(s) in this pathway	Effect on cell growth	Effect on stress signaling
PP2Cα	AMPK	AMPK	—	↓
	JNK	MKK4, MKK7	—	↓
	p38	p38, MKK3b, MKK6b	—	↓
	CDK	Cdk2, Cdk6	↓	—
	p53	MDM2	↓	↑
	TGFβ	Smad2, Smad3	↓↑*	—
	Wnt	LEF-1	↑	—
PP2Cβ	JNK	MKK4, MKK7, TAK1	—	↓
	p38	MKK3b, MKK6b	—	↓
	NF-κB	IKKβ	↓	↓
	CDK	Cdk2, Cdk6	↓	—
	p53	MDM2	↓	↑
	Bcl-xL	BAD	↓	—
PP2Cδ	p38	p38	—	↓
	p53	p53, p38, ATM	↑	↓
	ATM	ATM	↑	↓
	Chk	Chk1, Chk2	↑	—
	BER	UNG2	—	↓
	RSK	RSK1—4, MSK1—2	↓	↓
	INK4A	p38	↑	—
	ARF	p38	↑	—
PP2Cε	JNK	TAK1, ASK1	—	↓
PP2Cζ	SUMO	UBC9	—	—
	CAMK	CAMKII	—	—
CaMKP	JNK	PAK	—	↓
	FAS	F1A	↓	—
CaMKP-N	CAMK	CAMKIV, CAMKII-N	—	—
	JNK	PAK	—	↓
ILKAP	Wnt	ILK, GSK3β	↓	—
PHLPP	Akt	Akt	↓	—

↓ Indicates that the (over-)expression of the respective phosphatase results in an inactivation of the indicated pathway, ↑ indicates an activation of the pathway, and * indicates that the impact (of inhibiting TGFβ signaling) on cell growth is context-dependent.

Based on the experimental evidence obtained thus far, four type 2C phosphatases can be expected to possess tumor-suppressing properties. First, because it upregulates the expression and the activity of p53, because it reduces the activities of the growth-promoting cyclin-dependent kinases Cdk2 and Cdk6, and because it inhibits TGFβ-signaling, PP2Cα is likely to be a tumor suppressor protein. Second, as PP2Cα, PP2Cβ has been shown to be able to activate p53, and to inhibit Cdk2 and Cdk6. In addition, it has been shown to dephosphorylate and activate the proapoptotic protein BAD, thereby enabling BAD to neutralize the antiapoptotic effects of Bcl-xL. And furthermore, by attenuating the activity of IKKβ, PP2Cβ inhibits NF-kB-mediated antiapoptosis. By means of the latter two mechanisms, PP2Cβ is expected to contribute to restoring the balance between apoptosis and antiapoptosis, which, in tumor cells, tends to favor antiapoptosis. Third, as it has been shown to be an important physiological inhibitor of the ILK1-GSK3β-Wnt pathway, the recently identified type 2C phosphatase ILKAP might represent a tumor suppressor protein. ILK1, *i.e.*, the direct target of ILKAP, is known to be overexpressed in various human malignancies, and the activation of the ILK1-GSK3β-Wnt pathway is known to result in an increase in proliferation and oncogenesis. By dephosphorylating ILK1 and by inhibiting the activity of the ILK1-GSK3β-Wnt pathway, ILKAP inhibits both the proliferation and the oncogenic transformation of cells, and it can therefore be expected to possess tumor-suppressing properties. And finally, fourth, PHLPP can be considered to act as a tumor suppressor protein. Based on the observation

that it has been shown to dephosphorylate and inactivate Akt, on the notion that the upregulation of Akt is considered to be a key cause of cancer, and on the fact that the loss of the genomic region containing the PHLPP gene is frequently observed in human colon cancer specimens, it seems to be reasonable to assume that PHLPP possesses tumor-suppressing properties.

As opposed to the tumor-suppressing properties of PP2Cα, PP2Cβ, ILKAP, and PHLPP, a substantial amount of evidence exists indicating that PP2Cδ is an oncogene; it has been shown to inhibit the activity of four different tumor suppressor proteins (p53, ATM, INK4A, and ARF), to inhibit the function of the checkpoint kinases Chk1 and Chk2, and to negatively affect base-excision repair. By means of the former mechanism, PP2Cδ enhances cell growth, cellular proliferation and oncogenic transformation, and by means of the latter two mechanisms, it prevents the eradication of cells that, like tumor cells, harbor genetic defects and chromosomal aberrations. In addition, amplifications of the genomic region containing the PP2Cδ gene have been observed in breast carcinomas, in ovarian carcinomas, in neuroblastomas and in medulloblastomas. And furthermore, chemical inhibitors of PP2Cδ have been shown to be able to decrease the proliferation rate of cancer cells *in vitro*, to increase the sensitivity of cancer cells towards chemotherapeutic treatment *in vitro*, and to inhibit the growth of xenograft tumors *in vivo*. Together, these findings clearly classify PP2Cδ as an oncogene, and they indicate that the inhibition of this type 2C phosphatase represents an interesting strategy for attempting to improve both the efficacy and the specificity of anticancer therapy.

Except for the PP2Cα- and PP2Cβ-mediated upregulation of p53, in all cases in which they have been linked to the control of the cellular stress response, type 2C protein phosphatases have been found to act as inhibitors of cellular stress signaling (Table 2). Figure 1 shows that at least four different PP2Cs are directly involved the regulation of the MAPK pathway, arguably the most important stress signaling pathway in eukaryotes. Interestingly, only those two MAPK pathways that are known to be involved in dealing with cellular stress have been linked to type 2C phosphatases; the JNK pathway has been shown to be inactivated by PP2Cα, by PP2Cβ and by PP2Cε, and the p38 pathway by PP2Cα, by PP2Cβ, by PP2Cδ and by PP2Cε (Figure 1). Evidence for an involvement of PP2Cs in the regulation of the third MAPK pathway, *i.e.*, the mitogen-activated ERK pathway, on the other hand, has not been obtained thus far. In addition to these notions, PP2Cα has been shown to attenuate the activity of the stress-activated AMPK (AMP-activated protein kinase) pathway, and CaMKP and CaMKP-N have been shown to inactivate the p21-activated kinase PAK, which is known to be one of the upstream activators of JNK. And furthermore, besides inactivating p38, PP2Cδ has been shown to dephosphorylate the P90 ribosomal S6 kinases RSK1–4 and the mitogen- and stress kinases MSK1–2, to inactivate p53 and ATM, and to dephosphorylate UNG2, thereby inhibiting (UNG2-mediated) base-excision repair. Based on these observations, it seems justified to conclude that type 2C protein phosphatases are putative physiological inhibitors of the cellular stress response.

Collectively, the experimental evidence obtained thus far convincingly demonstrates that type 2C protein phosphatases are important regulators of cell growth and cellular stress signaling. This indicates that the activation or the inhibition of these enzymes holds significant potential for improving the treatment of a variety of different diseases, ranging from ischemic stroke and myocardial infarction, to spinal cord injuries and cancer. Therefore, in the years to come, an in-depth analysis of the clinical potential of (agonists and antagonists of) type 2C protein phosphatases is strongly warranted.

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