The catalytic role of the M2 metal ion in PP2Cα

Chang Pan1,2*, Jun-qi Tang3,4*, Yun-fei Xu1,2, Peng Xiao1,3, Hong-da Liu1,2,3, Hao-an Wang5, Wen-bo Wang1,2-3, Fan-guo Meng6, Xiao Yu3,4 & Jin-peng Sun1,3,7

PP2C family phosphatases (the type 2C family of protein phosphatases; or metal-dependent phosphatase, PPM) constitute an important class of signaling enzymes that regulate many fundamental life activities. All PP2C family members have a conserved binuclear metal ion active center that is essential for their catalysis. However, the catalytic role of each metal ion during catalysis remains elusive. In this study, we discovered that mutations in the structurally buried D38 residue of PP2Cα (PPM1A) redefined the water-mediated hydrogen network in the active site and selectively disrupted M2 metal ion binding. Using the D38A and D38K mutations of PP2Cα as specific tools in combination with enzymology analysis, our results demonstrated that the M2 metal ion determines the rate-limiting step of substrate hydrolysis, participates in dianion substrate binding and stabilizes the leaving group after P-O bond cleavage. The newly characterized catalytic role of the M2 metal ion in this family not only provides insight into how the binuclear metal centers of the PP2C phosphatases are organized for efficient catalysis but also helps increase our understanding of the function and substrate specificity of PP2C family members.
lack of an efficient strategy to specifically remove or exchange the metal ion from one specific site. In our recent studies, we discovered that cadmium is a potent and specific inhibitor of PP2C family phosphatases that acts by binding to the M1 site of the binuclear metal center (Fig. 1a). The specific reduction in the negative charge of the M1 site coordinated residue D441 in PPM1G altered the metal inhibition profile. These results suggested that the M1 metal ion is required for catalysis and determines the metal ion inhibitory profile of PP2C phosphatase. By contrast, the contribution of the M2 site metal ion during substrate hydrolysis has not been investigated. Therefore, we set out to delineate the catalytic role of the M2 metal ion by specifically mutating its surrounding residues.

The mutations of conserved D38 in PP2Cα (the product of the PPM1A gene) to Ala or Lys impaired the protein’s cellular functions and decreased its phosphatase activity. Moreover, crystallographic analysis uncovered that these D38 mutants disrupted Mn2+ binding at the M2 metal ion site. Our study provides the exact roles of the M2 metal ion during catalysis, which will help not only in understanding the catalytic properties of PP2C family members but also extend our knowledge about metal-mediated metalloenzyme catalysis.

**Results**

**The M2 metal ion binding site in the PP2C family.** The PP2Cα is the prototype PP2C family phosphatase for which the catalytic
mechanisms and cellular functions have been well characterized. Therefore, we chose PP2C\textsubscript{a} as a model for investigating the catalytic role of the M2 metal ion. In the crystal structure of PP2C\textsubscript{a}, the M2 metal ion is directly coordinated to the primary chain carbonyl of the Gly61 and the side-chain carboxylate group of Asp60 (Fig. 1a). Both Asp60 and Gly61 are invariant residues among all PP2C family members in \textit{Homo sapiens}. Asp60 coordinated to both the M1 metal ion and the M2 metal ion, and an Asp60 mutation to Ala or Asn decreased the phosphatase activity more than 30-folds (Fig. 1b). In addition to these direct interactions, the M2 metal ion of PP2C\textsubscript{a} also indirectly interacts with Glu37 and Asp38 through water-mediated hydrogen bonds (Fig. 1a). Neither Asp38 nor Glu37 is involved in direct M1 metal ion coordination, and both residues are conserved among most PP2C family members (Fig. 1b and Supplementary Fig. S1). In comparison with Glu37, Asp38 is more conserved along a broader scope of different phosphatases (Supplementary Fig. S1). Therefore, we altered the side chain properties of Asp38 to see whether it can provide a specific tool for investigating M1 metal ion functions.

The D38 mutants impair PP2C\textsubscript{a}-regulated signaling pathways in cells. We made a D38A mutation in PP2C\textsubscript{a}, which eliminated the D38 side chain and abolished the indirect interaction between D38 and the M2 metal ion. To reverse the negative charge of the D38, we also made a D38K mutant to see the mutating effect.

Previous studies have demonstrated that PP2C\textsubscript{a} is a potent negative regulator of nerve growth factor (NGF)- or epidermal growth factor (EGF)-induced extracellular regulated protein kinases (ERK) phosphorylation\textsuperscript{19,22,23} and osmotic stress-induced c-Jun N-terminal kinases (JNK) phosphorylation\textsuperscript{24}. ERK and JNK are members of mitogen-activated protein kinases, which are key regulators of multiple signaling pathways. Similar to previous studies, the overexpression of the PP2C\textsubscript{a} wild type (PP2C\textsubscript{a}-WT) almost abolished EGF-induced ERK1/2 phosphorylation in HEK293 cells (Fig. 2a and 2b). However, the overexpression of an equal amount of PP2C\textsubscript{a}-D38A mutant as the wild type only slightly decreased the EGF-induced ERK phosphorylation, and the overexpression of the PP2C\textsubscript{a}-D38K mutant has little effect on the ERK phosphorylation increase (Fig. 2a and 2b). Accordingly, the overexpression of the PP2C\textsubscript{a}-WT, but not the D38A or D38K mutants, significantly blocked sorbitol-induced JNK phosphorylation in U251 cells (Fig. 2c and 2d). Taken together, these results demonstrated that the D38 mutants impaired PP2C\textsubscript{a}-regulated signaling pathways in cells.

The D38 mutants of PP2C\textsubscript{a} decreased the enzyme activity for pNPP, phospho-peptide and phospho-protein substrates. To investigate}

---

**Figure 2** | D38A and D38K mutants impaired the cellular functions of PP2C\textsubscript{a}. (a) The cellular effects of PP2C\textsubscript{a}-WT, D38A and D38K mutant overexpression on EGF-induced ERK phosphorylation in HEK293 cells. The HEK293 cells were cultured in DMEM containing 1% penicillin/streptomycin, 10% fetal bovine serum and 25 mM glucose at 37°C and 5% CO\textsubscript{2}. The cells were transfected with the full-length wild-type PP2C\textsubscript{a} and its mutants (D38A and D38K). After 36 hours of transfection and 12 hours of starvation, the HEK293 cells were stimulated with 5 ng/ml EGF for 5 min at 37°C. The levels of ERK1/2 phosphorylation were monitored using a phospho-ERK1/2-pT\textsuperscript{202}pY\textsuperscript{204} antibody. Total ERK1/2 was used as a loading control. The western blot shows a representative result from at least three experiments. (b) Statistical analysis of Fig.2a. *: P < 0.05, cells transfected with PP2C\textsubscript{a}-WT compared with control cells. #: P < 0.05, cells transfected with D38A or D38K compared with cells transfected with PP2C\textsubscript{a}-WT. The data showed are the average of at least three independent experiments. (c) The cellular effects of PP2C\textsubscript{a}-WT, D38A and D38K on sorbitol-induced JNK phosphorylation in U251 cells. The U251 cells were cultured in medium similar to HEK293 cells. 36 hours after transfection, the U251 cells were starved for 12 hours and then stimulated with 0.4 M sorbitol for 30 min at 37°C. The phospho-JNK-pT\textsuperscript{221}pY\textsuperscript{223} levels were monitored by western blot. The data shows a representative result from at least three experiments. (d) Statistical graph of Fig.2c. *: P < 0.05, cells transfected with PP2C\textsubscript{a}-WT compared with control cells. #: P < 0.05, cells transfected with D38A or D38K compared with cells transfected with PP2C\textsubscript{a}-WT. The data showed are the average of three independent experiments at least.
whether D38 mutants impaired the cellular functions of PP2Cα by affecting its phosphatase activities, we measured the enzyme kinetics of the PP2Cα-WT, D38A and D38K mutants towards the small artificial substrate pNPP, the phospho-peptides derived from pp-ERK (TGFpT202EpY204VATR), and pp-P38 (TDDEMpT180GpY182VAT) (Table 1). For the small artificial substrate pNPP, the $k_{\text{cat}}$ of D38A and D38K decreased by 2 ~ 4-fold, and the $k_{\text{cat}}/K_m$ decreased by approximately 5-fold in comparison with the PP2Cα-WT (Table 1). For the phospho-peptide, the $k_{\text{cat}}$ of D38A and D38K decreased by 3 ~ 45-fold, and the $k_{\text{cat}}/K_m$ decreased by 8 ~ 300-fold (Table 1). We further monitored the phosphatase activity of these mutants towards recombinant phospho-ERK protein in the test tube. As shown in Fig. S2, D38 mutants significantly decreased their activity towards the phospho-ERK protein substrate. Taken together, the D38A and D38K mutants of PP2Cα decreased the phosphatase activity toward a panel of small physiological substrates.

The crystal structures revealed that D38 mutants impaired metal ion binding at the M2 site. To further elucidate the structural basis of the impaired function and catalysis of D38 mutants, we solved the crystal structures of PP2Cα-D38A and D38K with Mn$^{2+}$ at 2.0 Å and 1.85 Å, respectively, and compared these structures with the PP2Cα-WT that was crystallized under similar conditions (1.95 Å) (Supplementary Table S1 and Fig. 3a–c).

In the Fo-Fc-annealing OMIT map, the electron density for the M1 metal ion can be unambiguously assigned in all three PP2Cα crystal structures. In addition, the electron density for the side chains of D38, A38 and the partial side chain of K38 are also unambiguously assigned in the PP2Cα-WT, PP2Cα-D38A and D38K crystal structures, respectively (Fig. 3a–3c). However, in contrast to the crystal structure of the PP2Cα-WT, the electron density for the M2 metal ion and the phosphate can’t be clearly located in the D38A or D38K crystal structures (Fig. 3a–3c).

Specifically, one water (WAT11) between the D38 and D60 in the PP2Cα-WT crystal structure was replaced by two waters (WAT8 and WAT248) in the solvent channel between the A38 and D60 in the D38A crystal structure, which form specific H-bond with the main chain amide of G61 (Fig. 4a–4b and S3a). The positions of these two waters preclude M2 metal ion binding to the D38A mutant protein. In the crystal structure of the D38K mutant, the bridging water between the M2 metal ion and the D38 in the PP2Cα-WT was pushed outward by 0.7 Å because of the longer K38 side chain (Fig. 4a, 4c and S3b). The relocated water (WAT145) forms a hydrogen bond with the side chain of K38, which disabled M2 metal ion binding. Therefore, the crystal structures of PP2Cα-D38 mutants revealed that the D38A or D38K mutants of PP2Cα abolished the M2 metal ion binding by altering the H-bond network of the PP2Cα active site.

### Table 1 | Kinetic constants of the activity of PP2Cα and its mutants at D38 position (D38A and D38K) toward pNPP and phospho-peptides

|        | $k_{\text{cat}}$ (s$^{-1}$) | $K_m$ (mM) | $k_{\text{cat}}/K_m$ (M$^{-1}$s$^{-1}$) | Ratio of $k_{\text{cat}}/K_m$ (wild-type/mutant) |
|--------|--------------------------|------------|------------------------------------|-----------------------------------------------|
| **P2Cα** |                          |            |                                    |                                               |
| D38A   | 2.52 ± 0.06              | 4.05 ± 0.25| 622 ± 23                           | 1                                             |
| D38K   | 1.46 ± 0.05              | 12.2 ± 1.08| 120.3 ± 8.7                        | 5.17                                          |
|        | 0.68 ± 0.03              | 4.88 ± 0.68| 139 ± 15                           | 4.49                                          |
| **P2Cα** |                          |            |                                    |                                               |
| D38A   | 0.36 ± 0.03              | 5.5 ± 1.1  | 6.55 ± 1.46                        | 1                                             |
| D38K   | 0.12 ± 0.01              | 15.8 ± 2.0 | 0.76 ± 0.29                        | 8.62                                          |
|        | 0.008 ± 0.0002           | 37.5 ± 6.9 | 0.02 ± 0.002                       | 327.5                                         |
| **P38** |                          |            |                                    |                                               |
| pT180pY182 |                      |            |                                    |                                               |
| D38A   | 0.26 ± 0.03              | 23.9 ± 4.1 | 1.09 ± 0.20                        | 1                                             |
| D38K   | 0.08 ± 0.01              | 31.4 ± 6.0 | 0.25 ± 0.07                        | 4.36                                          |
|        | 0.002 ± 0.0004           | 17.7 ± 2.6 | 0.01 ± 0.002                       | 109                                           |

The assays were performed in the Bis-Tris/Tris-acetate buffer (0.05 M Tris, 0.05 M Bis-Tris, 0.1 M acetate) pH 7.0 containing 10 mM Mn$^{2+}$ and 1 mM DTT at 25°C. The $k_{\text{cat}}$, $K_m$ and $k_{\text{cat}}/K_m$ values were fitted to the equation 2 with GraphPadPrism5 software. The data were repeated thrice at least.

The loss of metal ion binding at the M2 site decreased dianion binding. In the PP2Cα-WT crystal structure, the M2 metal ion forms two water-mediated hydrogen bonds with the oxygen in the phosphate and contributes to substrate dianion binding (Fig. 1a). The loss of the M2 metal ion in the PP2Cα-D38A and D38K mutants may have disturbed the basic nature of the active site and disrupted the water-mediated H-bonds, thus decreasing dianion phospho-substrate binding during intermediate enzyme-substrate formation. Therefore, we measured the inhibition constants of dianion sulfate toward PP2Cα-WT and D38 mutants. Accordingly, the D38A decreased sulfate binding by 4-fold, and D38K decreased the sulfate binding by 10-fold (Table 2). The decreased sulfate binding in D38A and D38K mutants are in agreement with the observation that there are no obvious electron density for phosphate in the crystal structures of D38A and D38K (Fig. 3a–c). Taken together, these results demonstrated that the M2 site metal ion contributes to phospho-substrate binding, which is the initial step of enzyme-substrate complex formation.

The pH dependence of catalysis revealed that the D38A and D38K mutants affected the rate-limiting chemical step. A bell-shaped curve was observed for the pH dependence of $k_{\text{cat}}/K_m$ toward pNPP as catalyzed by PP2Cα-WT in the presence of Mg$^{2+}$ as the activating metal ion, suggesting the presence of the general acid-base catalytic mechanism (Fig. 3a). In consistence with previous studies, the pH dependence of $k_{\text{cat}}/K_m$ of PP2Cα-WT toward pNPP indicated that one group must be unprotonated (pK$_a$ ~ 7.54) and one group must be protonated (pK$_a$ ~ 8.53) (Fig. 5b)$.^{18,20,21}$ Similar to PP2Cα-$\alpha$.
WT, the $k_{cat}/K_m$ of the D38A and D38K mutants increase from pH 5 to pH 7, indicating the presence of a general base during the catalysis. However, the basic limbs of the D38A and D38K catalyzed pNPP hydrolysis were flat, suggesting an inefficient general acid catalysis for these two mutations.

The leaving group dependence of the PP2Ca-WT and the D38A and D38K mutants. We therefore further evaluated the effects of the D38 mutants on the general acid catalysis by determining the dependence of the reaction on the nature of the leaving group with a Bronsted plot analysis (Fig. 5c). The $\beta_{lg}$ values were derived from a linear least-squares fitting of the appropriate Bronsted plots. When the $\beta_{lg}$ value of the $k_{cat}$ is approximately $-1$, then there is no general acid to stabilize the leaving group of the phenolic oxygen from the substrate. By contrast, when the $\beta_{lg}$ value of $k_{cat}$ is approximately 0, then an efficient general acid is present$^{25-29}$. As a result, the $\beta_{lg}$ value...
Previous studies showed that the H62 of PP2Cα might act as a general acid during the cleavage of the P-O bond. In agreement with their findings, the $k_{cat}$ value for $k_{cat}$ for H62N was $0.54$, suggesting an impaired general acid catalysis. We next measured the $k_{cat}$ value for the $k_{cat}$ of the H62N/D38A double mutant to see whether the D38A mutant impaired the general acid catalysis by disturbing the H62 conformation. As a result, the $k_{cat}$ value for the $k_{cat}$ of H62N/D38A is $0.7$, which is significantly lower than that of both H62N and D38A (Fig. 5d and 5e). Therefore, H62 and D38 independently participate in general acid catalysis, in which they help to provide a potentially positive charged proton to stabilize the negative charge of the oxygen anion after phospho-oxygen bond cleavage. In conclusion, the M2 metal ion plays an important role in stabilizing the negative charge developed by the leaving group of the PP2C substrates.

### Discussion

The catalytic role of the M2 metal ion in PP2C family phosphatases is not defined, partially because of the lack of specific tools for disrupting M2 metal ion binding in the PP2C active site. Both the D38 and E37 of the PP2Cα are conserved among PP2C family members and specifically interact with the M2 metal ion through water-mediated hydrogen bonds (Fig. 1a and 1b). Unlike E37, which is exposed to the solvent, D38 is buried inside the PP2Cα structure and forms an important hydrogen bond with a structural water inside of the protein to stabilize the M2 metal ion binding (Fig. 3a). Our crystal structures revealed that the D38A and D38K mutations altered the water-mediated hydrogen bond network and disrupted M2 metal ion binding with the maintenance of the protein folding and other active site conformations, which provide specific tools to investigate the functional role of the M2 metal ion among PP2C phosphatases (Fig. 3b–c and Supplementary Table S2). Moreover, because the D38 is not surface-exposed, it does not directly participate in substrate hydrolysis.

The binuclear metal center is required for the PP2Cα family of phosphatases, and the binding of the active site with different metal ions either activates or inhibits the activities of PP2C family phosphatases. However, the catalytic roles for each metal ion remain elusive. In our previous study, we confirmed that cadmium specifically blocked PP2C phosphatase activity by binding to the M1 metal ion site, and the chemical property of the coordinated residue of the M1 metal ion determines the inhibitory specificity of the metal ions. This finding was confirmed by scanning the activating or inhibitory profile of a panel of metal ions towards PP2Cα and PP2Cγ, together with an analysis of a series of PP2C mutants. These results suggested that the M1 metal ion is one determinant for catalysis. Using specific M2 metal ion-coordinated residue D38A and D38K mutations, we discovered that the M2 metal ion participates in the initial step of dianion phospho-substrate binding, affects the rate-limiting chemical step and is involved in the neutralization of the negative charge in the leaving group according to a combined enzymology analysis. Interestingly, the D38A and the previously proposed general acid known as H62 work independently to stabilize the leaving group as revealed by Brožnsted plot analyses. It is likely that both H62 and the M2 metal ion-coordinated water can function as general acids during catalysis.

Besides the binuclear metal center, recent studies demonstrated that a third metal ion is required for the catalysis of PP2Cα and PP2Cγ towards phospho-peptide substrates. Biochemical studies and mutating effects suggest that the third metal ion M3 interact with residues of D146, D243 and D239 of PP2Cα. However, the exact

### Table 2 | Inhibition constant of $SO_4^{2-}$ toward PP2Cα, D38A and D38K

| $SO_4^{2-}$ | $K_i$ (mM) | $SO_4^{2-}$ | $K_i$ (mM) | $SO_4^{2-}$ | $K_i$ (mM) |
|-------------|------------|-------------|------------|-------------|------------|
| PP2Cα       | 15.4 ± 1.3 | D38A        | 61.8 ± 5.1 | D38K        | 213.2 ± 56.8 |

The assays were performed in the buffer containing 0.05 M Tris, 0.05 M Bis-Tris, 0.1 M acetate and 10 mM Mg$^{2+}$ at 25°C and pH 7.0 with varying concentrations of pNPP and Na$_2$SO$_4$. The data were fitted to the equation 5 and were analyzed by software GraphPadPrism5. The data showed were repeated three times at least.
mechanism of M3 metal ion participating in PP2Ca catalysis is still lacking and the third metal ion is missing in the crystal structures of PP2Ca wild type or mutants due to the low pH of crystallization condition. Further structural and enzymologic studies are required to fully understand the catalytic roles of metal ions in the PP2Ca family phosphatases.

Taken together, we have identified the specific catalytic roles of the M2 metal ion during catalysis, there could be a general mechanism that is used by other metalloenzymes that provides clues for the design of new PP2C inhibitors and de-novo enzymes.

Methods

Materials. Para-nitrophenyl phosphate (pNPP), β-naphthyl phosphate, phenyl phosphate, 4-methylumbelliferyl phosphate and polyethylene glycol (PEG mol. wt. 8000) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). O-phospho-L-tyrosine was purchased from Sigma. Peptides including ppERK2 (TGElpT202EpY204VATR) and pp-P38 (TDDEMpT180GpY182VAT) were obtained from China Peptides Co. (China). The BIOMOL green TM reagent for phosphate detection (BML-AK111) was purchased from Enzo Life Sciences. The Flag tag

Figure 5 | The effects of the pH and the leaving group pKₐ of substrates on the kcat/Km of wild-type PP2Ca and its mutants. (a) The kcat/Km values for PP2Ca-WT (red circle), D38A (green diamond), and D38K (blue square) were determined by varying the pNPP concentration at 40 mM Mg²⁺ and different pH values. The assays were performed in Tris/bis-Tris/acetate buffer with different pH values from 5.5 to 10 at 25°C. (b) The pKₐ, pKₐ and kcat/Km values generated from figure 5a. (c) The Brønsted plots for a PP2Ca-catalyzed hydrolysis of aryl phosphates; PP2Ca-WT (red circle), D38A (green diamond) and D38K (blue square). Substrates pNPP (pKₐ = 7.1), 4-methylumbelliferyl phosphate (pKₐ = 7.8), β-naphthyl phosphate (pKₐ = 9.38), phenyl phosphate (pKₐ = 9.99), O-phospho-L-tyrosine (pKₐ = 10.07) and phosphoserine (pKₐ = 14.2) were used. The assays were performed under the same conditions as the pH dependence experiments (Fig. 5a). (d) Brønsted plots for the H62N (blue circle) and D38A/H62N double mutations (green triangle). (e) β₁⁺ values were obtained by fitting Brønsted plots from figures 5c and 5d.
DYKDDDDK) antibody, GAPDH antibody, pp-P38 MAPK (pT180/pY182) antibody and pp-P44/42 MAPK (ERK) antibody were purchased from Sigma. Anti-Flag, His tag antibody, and antibodies against GADPH, pp-P38 MAPK (pT180/pY182), pp-P44/42 MAPK (ERK) were purchased from Cell Signaling Technology. Ni-NTA agrose was purchased from Roche. All other materials are from Sigma company. All other materials are from Sigma company.

 Constructs and site-directed mutagenesis. The full-length human PP2Cα cDNA was a gift from Professor Patricia T. W. Cohen (MRC Protein Phosphorylation Unit). The Flag-tagged PP2Cα construct used for the cellular assays was described previously. The PP2Cα mutants (D38A, D38K, H62N and H62N/D38A) were generated with a QuickChange mutagenesis kit. PAGE-purified oligonucleotide primers were purchased from the Beijing Genomics Institute (China) and all mutations were verified by DNA sequencing.

 Protein purification. The expression and purification of PP2Cα with a N-terminal His tag in addition to its mutants have been described previously25,26. In brief, BL21 E. coli cells were transformed with PP2Cα plasmids and cultured at 37°C. The cultures were induced with 0.5 mM IPTG overnight at 25°C and then pelleted by centrifugation at 3,200 g for 20 min. The cell pellets were re-suspended in 60 ml of ice-cold “His buffer” (20 mM Tris, pH 8.0, and 300 mM NaCl) and crushed three times with a high-pressure cellcracker. The lysates were then centrifuged at 13,000 g for 40 min at 4°C. The supernatant was centrifuged at 1 ml of Ni beads (Ni-NTA agarose) for 1.5 h. The Ni beads were then washed with 100 ml of ice-cold “His buffer” at 4°C. The bound His-PP2Cα protein was finally eluted with a buffer containing 20 mM Tris pH 8.0, 300 mM NaCl and 100 mM imidazole. Before being frozen at ~80°C, the proteins were exchanged for a buffer containing 0.05 M Tris, 0.05 M bis-Tris, 0.1 M acetic acid, pH 8.0, 1 mM DTT and 1 mM Mn2+27. The purity of the protein was examined by Coomassie brilliant blue staining after SDS-PAGE. The protein concentration was measured at OD260/OD280 in a spectrophotometer30. The molar absorption coefficient of the enzyme-substrate complex in the rate-limiting step, and [H] is the concentration of H+ ions.

Data analysis. All data were presented as the means ± SEM. Statistical comparisons were performed using one- or two-way ANOVA tests in GraphPad Prism5. A multiple regression equation was performed using the inorganic phosphate detection assay at pH 7.0. The log(ka) values were plotted against the pKα of leaving-group of the substrates to acquire the Brönsted plots. The βp values for ka were obtained from fitting the data to a linear least-square regression equation.

Enzyme Kinetics. To determine the kinetic parameters of κcat and κcat/KM, the reactions were performed in a reaction buffer (0.05 M Tris, 0.05 M bis-Tris, and 0.1 M acetic acid pH 7.0) containing suitable divalent Mn++ at 25°C. The initial velocities were measured at appropriate pNPP concentrations and saturating levels of Mn++. The reactions were terminated by 500 mM EDTA (pH 10.0) and assayed by measuring the absorbance of pNP at 405 nm. For enzyme assays using phosphopeptides as substrates, an inorganic phosphate assay with an end-point reading at 620 nm was determined for phosphate release. The KM, kcat, and KC values were obtained by fitting the data to equation 2.

\[ V = \frac{V_{\text{max}} \times [S]}{(K_M + [S])} \]  

(eq. 2)

To measure the pH dependence of phosphatase catalysis, the kcat and kcat/KM of PP2Cα (wild-type, D38A and D38K) were obtained at various pH values (Hepes-Tris-Tris-acetate buffer) with pNP as the substrate at a saturating Mg2+ level (40 mM for PP2Cα). The pH data were fitted to Equation 3 and Equation 4. The kcat/KM values means the pH-independent value of kcat/KM, and ka are the apparent ionization constants of the enzyme-substrate complex in the rate-limiting step, and [H] is the concentration of H+ ions.

Crystallography. Crystals of PP2Cα protein were performed as previously described. Briefly, PP2Cα protein without N-terminal His tags (wild-type, D38A, and D38K) were crystallized at 15 mg/ml buffered to pH 4.0-4.5 at 4°C. Data collection and structure determination. Diffraction data were collected in Shanghai Synchrotron Radiation Facility (BL17U). Data for PP2Cα, (wild type, D38A and D38K) were processed using the HKL2000 program and their structures were determined by molecular replacement with Phaser in the CCP4 software package. The crystals all belong to the P32121 space group. A single chain of the PP2Cα was used as the initial search model. Further refinements of the crystals were processed by the PHENIX program with iterative manual building in COOT. To reduce the phase biases during further refinement, composite omitted maps were built and density modification was executed. Ramachandran plots were calculated with PROCHECK and COOT. The statistics for the final solved structures are shown in Table S1.

Cell culture, transfection, and western blotting. Human kidney 293 cells and U251 cells were cultured in DMEM including 1% penicillin/streptomycin, 10% fetal bovine serum and 25 mM glucose under a humidified atmosphere containing 5% CO2 at 37°C. HEK293 and U251 cells were transfected with the full-length wild-type PP2Cα and its mutants (D38A and D38K) with an N-terminal Flag tag with PEI (Polysciences). Thirty-six h after the transfection, the cells were starved for 12 h and then stimulated with 5 ng/ml EGF for 5 min (HEK293 cells) and 0.4 M sorbitol for 30 min (U251 cells) at 37°C. The cells were subsequently washed twice with cold phosphate-buffered saline (PBS) and then harvested with lysis buffer (50 mM Tris- HCl, pH 7.5, 0.5% NP-40, 0.25% sodium deoxycholate, 1% nonidet P-40, 1 mM EDTA, 50 mM NaF, 10% (v/v) glycerol, 1 mM Na2VO4, 5 mM iodoacetamide (IAA) and protease inhibitor cocktail). An irreversible peptides inhibitor, IAA can react with sulfhydryl of cysteine residues and prevent the formation of disulfide bonds. The cell lysates were lysed on ice for 30 min and then centrifuged at 13,000 g for 30 min at 4°C to collect the supernatant. The protein sample concentrations were measured with a BCA Protein Quantitation Kit. The same amount of 2 × SDS loading buffer was added to lysate proteins and then boiled for 10 min. The protein samples were then subjected to western blotting. Proteins were transferred to nitrocellulose membranes after running SDS-PAGE and incubated in appropriate primary antibodies at 4°C overnight, and then incubated in HRP-conjugated secondary antibody. Reactive bands were detected with Western Chemiluminescent HRP Substrate (Millipore). The Image software was used to quantify the signals from western blots and a standard curve of Western blot with linear increase of the target protein amount was used to correct the potential deviations.

To measure the pH dependence of phosphatase catalysis, the kcat and kcat/KM of PP2Cα (wild-type, D38A and D38K) were obtained at various pH values (Hepes-Tris-Tris-acetate buffer) with pNP as the substrate at a saturating Mg2+ level (40 mM for PP2Cα). The pH data were fitted to Equation 3 and Equation 4. The kcat/KM values means the pH-independent value of kcat/KM, and ka are the apparent ionization constants of the enzyme-substrate complex in the rate-limiting step, and [H] is the concentration of H+ ions.

In vitro dephosphorylation of phospho-ERK2 protein. The phosphatase activity was measured in vitro with phospho-P2Cα in vitro was performed as previously described14,15. To determine the kinetic parameters of kcat and KM, the reactions were performed in a reaction buffer (0.05 M Tris, 0.05 M bis-Tris, and 0.1 M acetic acid pH 7.0) containing suitable divalent Mn++ at 25°C. The initial velocities were measured at appropriate pNPP concentrations and saturating levels of Mn++. The reactions were terminated by 500 mM EDTA (pH 10.0) and assayed by measuring the absorbance of pNP at 405 nm. For enzyme assays using phosphopeptides as substrates, an inorganic phosphate assay with an end-point reading at 620 nm was determined for phosphate release. The KM, kcat, and KC values were obtained by fitting the data to equation 2.

\[ \frac{V}{V_{\text{max}}} = \frac{K_M + [S]}{(K_M + [S])} \]  

(eq. 3)

To measure the leaching group dependency for the PP2C phosphatase-catalyzed reaction, the dephosphorylation rates were analyzed for small artificial substrates with different pKα values as previously described26. The ki values for the leaching substrates (pNPP (7.1), 4-methylumbelliferyl phosphate (7.8), β-naphthyl phosphate (9.38), phenyl phosphate (9.99), o-phospho-L-tyrosine (10.07) and phosphoserine (14.13)) were analyzed using the inorganic phosphate detection assay at pH 7.0. The log( ki ) values were plotted against the pKα of leaving-group of the substrates to acquire the Brönsted plots. The βp values for ki were obtained from fitting the data to a linear least-square regression equation.

The KM, kcat, and KC values were obtained by fitting the data to equation 2.

\[ \frac{V}{V_{\text{max}}} = \frac{K_M + [S]}{(K_M + [S])} \]  

(eq. 4)
2. Ma, Y. et al. Regulators of PP2C phosphatase activity function as aspartic acid sensors. *Science* **324**, 1064–1068, doi:10.1126/science.1172408 (2009).

3. Takeuchi, J. et al. Designed aspartic acid analogs as antagonists of PYL–PP2C receptor interactions. *Nat Chem Biol* **10**, 477–482, doi:10.1038/nchembio.1524 (2014).

4. Shi, Y. Serine/threonine phosphatases: mechanism through structure. *Cell* **139**, 468–484, doi:10.1016/j.cell.2009.10.006 (2009).

5. Ota, I. M. & Mapes, J. Targeting of PP2C in budding yeast. *Methods Mol Biol* **365**, 309–322, doi:10.1007/978-59745-267-X309 (2007).

6. Cheng, A., Ross, K. E., Kaldis, P. & Solomon, M. J. Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases. *Genes Dev* **13**, 2946–2957 (1999).

7. Kleiblova, P. et al. Gain-of-function mutations of PPM1D/Wip1 impair the p53-dependent G1 checkpoint. *J Cell Biol* **201**, 511–521, doi:10.1083/jcb.201210031 (2013).

8. Filipponi, D., Muller, J., Emelyanov, A. & Bulavin, D. V. Wip1 controls global heterochromatin silencing via ATM/BRAF1-dependent DNA methylation. *Cancer Cell* **24**, 528–541, doi:10.1016/j.ccc.2013.08.022 (2013).

9. Rourke, E. et al. Mosaic PPM1D mutations are associated with predisposition to breast and ovarian cancer. *Nature* **493**, 406–410, doi:10.1038/nature12175 (2013).

10. Zhang, L. et al. Exome sequencing identifies somatic gain-of-function PPM1D mutations in breast invasive glandular tumors. *Nat Genet* **46**, 723–730, doi:10.1038/ng.2995 (2014).

11. Lu, G. et al. Protein phosphatase 2Cm is a critical regulator of branched-chain amino acid catabolism in mice and cultured cells. *J Clin Invest* **119**, 1678–1687, doi:10.1172/JCI8151 (2009).

12. Oyarzabal, A. et al. A novel regulatory defect in the branched-chain alpha-keto acid dehydrogenase complex due to a mutation in the PPM1K gene causes a mild variant phenotype of maple syrup urine disease. *Hum Mutat* **34**, 355–362, doi:10.1002/humu.22242 (2013).

13. McNamara, R. P., McCann, J. L., Gudipaty, S. A. & Orso, I. Transcription factors mediate the enzymatic disassembly of promoter-bound 7SK snRNPs to locally recruit P-TEFb for transcription elongation. *Cell Rep* **5**, 1256–1268, doi:10.1016/j.celrep.2013.11.003 (2013).

14. Khoronenkova, S. V. Regulation of serine/threonine phosphatase-mediated catalysis. *Biochem J* **378**, 421–433, doi:10.1042/BJ20050365 (2004).

15. Allemand, E., Hastings, M. L., Murray, M. V., Myers, M. P. & Krainer, A. R. Alternative splicing regulation by interaction of phosphatase PP2Cgamma with G1A (PPM1A) plays a role in the differentiation and survival processes of nerve cells. *PLoS One* **7**, e32438, doi:10.1371/journal.pone.0032438 (2012).

16. Takekawa, M., Maeda, T. & Saito, H. Protein phosphatase 2C inhibits the human stress-responsive p38 and JNK MAPK pathways. *EMBO J* **17**, 4744–4752, doi:10.1093/emboj/17.16.4744 (1998).

17. Pedersen, A. K. et al. Residue 182 influences the second step of protein-tyrosine phosphatase-mediated catalysis. *Biochem J* **378**, 801–813, doi:10.1042/BJ20050365 (2004).

18. Das, A. K., Helps, N. R., Cohen, P. T. & Barford, D. Crystal structure of the protein phosphatase 2C at 2.0 A resolution. *Mol Cell* **5**, 309–322, doi:10.1016/j.molcel.2005.03.008 (2005).

19. Zhang, Z. Y., Malachowski, W. P., Van Etten, R. L. & Dixon, J. E. Altering the nucleophile specificity of a protein-tyrosine phosphatase-catalyzed reaction. Probing the function of the invariant glutamine residues. *J Biol Chem* **273**, 5484–5492 (1998).

20. Wang, W. Q. et al. Kinetic and mechanistic studies of a cell cycle protein phosphatase Cdc14. *J Biol Chem* **279**, 30459–30468, doi:10.1074/jbc.M402217200 (2004).

21. Tanoue, K. et al. Binding of a third metal ion by the human phosphatases PP2Calpha and Wip1 is required for phosphatase activity. *Biochemistry* **52**, 5830–5843, doi:10.1021/bi0400549 (2013).

22. Pace, C. N., Vaidos, F., Fee, L., Grimsley, G. & Gray, T. How to measure and predict molar absorbance coefficient of a protein. *Protein Sci* **4**, 2411–2423, doi:10.1002/pro.5560041220 (1995).

23. Ciopollotta, D. et al. PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* **486**, 549–553, doi:10.1038/nature11132 (2012).

24. Liang, S. D., Marmorstein, R., Harrison, S. C. & Ptashne, M. DNA sequence preferences of GAL4 and PPR1: how a subset of Zn2 Cys6 binuclear cluster proteins recognizes DNA. *Mol Cell Biol* **16**, 3773–3780 (1996).

25. Haegens, A. et al. NF-kappaB activation and polyubiquitin conjugation are required for pulmonary inflammation-induced diaphragm atrophy. *Am J Physiol Lung Cell Mol Physiol* **302**, L103–110, doi:10.1152/ajplung.00684.2011 (2012).

26. Huang, X., Zhou, B. & Zhang, Z. Y. Molecular determinants of substrate recognition in hematopoietic protein-tyrosine phosphatase. *J Biol Chem* **279**, 52150–52159, doi:10.1074/jbc.M407820200 (2004).

27. Zhao, Y. & Zhang, Z. Y. The mechanism of dephosphorylation of extracellular signal-regulated kinase 2 by mitogen-activated protein kinase phosphatase 3. *J Biol Chem* **276**, 32382–32391, doi:10.1074/jbc.M103692000 (2001).

**Acknowledgments**

This work was supported by grants from the National Key Basic Research Program of China (2013CB967070 to Dr. Xiao Yu; 2012CB910402 to Dr. Jin-Peng Sun); the National Natural Science Foundation of China (31270857 to Dr. Xiao Yu; 31100580 and 31470789 to Dr. Jin-Peng Sun), the Shandong Natural Science Fund for Distinguished Young Scholars (JQ201302 to Dr. Xiao Yu), the Fundamental Research Fund of Shandong University (2014JC029 to Dr. Xiao Yu), and Program for Changjiang Scholars and Innovative Research Team in University (IRT13028). The authors declare that there are no conflicts of interest.

**Author contributions**

J.P.S., X.Y. and C.P. designed all experiments; C.P. and J.Y.T. initiated the research; C.P., J.Y.T., Y.F.X., H.A.W. and W.B.W. performed biochemical, enzymology, cellular and molecular biology experiments; C.P., J.Y.T. and Y.F.X. corrected the crystal structure and analyzed data; J.P.S., X.Y. and C.P. wrote the paper; J.P.S. and X.Y. provided funding; F.G.M., X.Y. and J.P.S. discussed the results and provided essential reagents. All the authors reviewed the paper and agreed for publication.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pan, C. et al. The catalytic role of the M2 metal ion in PP2Cz. *Sci. Rep.* **5**, 8560; DOI:10.1038/srep08560 (2015).