

## PPP3CC feedback regulates IP3-Ca<sup>2+</sup> pathway through preventing ITPKC degradation

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## 1. ABSTRACT

*ITPKC*, a susceptibility gene of Kawasaki disease, encodes a kinase that negatively regulates intracellular Ca<sup>2+</sup> level and inhibits calcineurin-dependent activation of NFAT by phosphorylating IP3. In this study, we identified a novel ITPKC-interacting protein, namely PPP3CC, using yeast two-hybrid. This interaction was further confirmed by GST pull-down and co-immunoprecipitation assays, and fluorescent microscopy showed co-localization of both proteins in the cell cytoplasm. Our functional studies demonstrated that PPP3CC positively influences the protein level of ITPKC, likely by inhibiting phosphorylation of ITPKC and consequently preventing ITPKC from ubiquitin-mediated protein degradation which requires phosphorylation. Importantly, the protein level of PPP3CC negatively correlates with the cellular level of IP3, suggesting a regulatory role of PPP3CC in the IP3-Ca<sup>2+</sup> signaling pathway.

## 2. INTRODUCTION

ITPKC (inositol-trisphosphate 3-kinase C) is a member of the inositol 1,4,5-trisphosphate 3-kinase family (1, 2). ITPKC phosphorylates IP3 (Inositol trisphosphate), an important second messenger molecule which binds to IP3 receptors on the endoplasmic reticulum and stimulates the release of Ca<sup>2+</sup> from intracellular Ca<sup>2+</sup> store and the influx of extracellular Ca<sup>2+</sup> into the cells (3-5), resulting in the increases of intracellular Ca<sup>2+</sup> concentration. Then, Ca<sup>2+</sup> forms a complex with calmodulin to activate calcineurin, which dephosphorylates NFAT (Nuclear factor of activated T-cells) and promotes its nuclear localization to activate the expression of a number of immune response-related genes including IL-2 (6-8). As a negative regulator in the Ca<sup>2+</sup> signaling pathway (1, 2), ITPKC phosphorylates and inactivates IP3 to block the IP3-Ca<sup>2+</sup> channel opening, leading to down-regulation of intracellular Ca<sup>2+</sup> concentration and the downstream events of the IP3-Ca<sup>2+</sup> signaling pathway.

Abnormal expression of ITPKC caused by DNA polymorphism was correlated with occurrence of Kawasaki disease (3, 9), a rare autoimmune condition mainly in children under 5 years old featured by inflammation of the medium-sized blood vessels. 20-25% cases may have coronary injuries without effective intervening (10-12). So far IPKTC was the only susceptibility gene linked to Kawasaki disease with known biological functions (13).

PPP3CC (protein phosphatase 3, catalytic subunit, gamma isozyme) is a member of calcineurin catalytic subunits (14) previously documented as a positive regulator in the IP3-Ca<sup>2+</sup> signaling pathway, which catalyzes the dephosphorylation of NFAT and promotes its nuclear translocation to activate the expression of inflammation-related downstream genes. Notably, *PPP3CC* is a schizophrenia susceptibility gene located at 8p21.3 (15).

In this study, we identified PPP3CC as a novel interacting partner of ITPKC. Strikingly, PPP3CC reduces the phosphorylation level of ITPKC, prevents ITPKC from ubiquitin degradation, and stabilizes ITPKC protein level. Thus, our results assign a novel role to PPP3CC as a negative regulator in the IP3-Ca<sup>2+</sup> signaling pathway through preventing ITPKC degradation.

### 3. MATERIALS AND METHODS

#### 3.1. Plasmid construction

ORF of human *ITPKC* (GeneID: 80271) was amplified by PCR from human liver cDNA library (Clontech), further cloned into the yeast two-hybrid system vector pDBLeu (Invitrogen), into pGBKT7 vector (Clontech) to generate a His-tag fusion protein, into pCMV-HA and pEF-flag vector (Clontech) for expressing in mammalian cells.

ORF of full length human *PPP3CC* (Gene ID: 5533) was amplified from human fetal liver cDNA library and cloned into pPC86 vector, into pGEX-5X-1 vector (Amersham Pharmacia Biotech) to generate GST-tag fusion protein, into pCMV-Myc and pEGFP-C1 vector (Clontech) for mammalian cells.

#### 3.2. Yeast two-hybrid screen

Two-hybrid screen was performed in the ProQuest<sup>TM</sup> two-hybrid system (Invitrogen). pDBLeu-ITPKC was used as the bait to screen a human fetal brain cDNA library.

#### 3.3. GST pull down assay

The fusion protein GST-PPP3CC was expressed in *E. coli* BL21, induced by 0.1mM IPTG at 26°C for 2 h and then purified with glutathione-Sepharose4B beads according to the manufacturer's protocol (GE Healthcare). pGBKT7-Myc-ITPKC fusion protein was obtained by TNT Quick Coupled Transcription/Translation System *in vitro* (Promega). GST pull-down assay was performed as previously described protocol (16).

#### 3.4. Cell culture, transfection and immunoprecipitation

HEK293 and Hela cells were obtained from American Type Culture Collection. The cells were

maintained in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% Fetal Bovine Serum (Hyclone). The transfections were performed using Lipofectamine<sup>TM</sup> 2000 (Invitrogen) according to the manufacturer's protocol. Cells were treated with MG132 (Sigma) and CHX (Sigma) for the protein stability and ubiquitination assay.

#### 3.5. Subcellular localization assay

HeLa cells were washed with PBS and fixed with 4% paraformaldehyde (pH=7.4) in PBS for 20 min at room temperature. After permeabilization with 0.5% Tween20 in PBS 10 min. Cells were stained with Hoechst33342 (Sigma) for 15 min at 37°C. Fluorescent images were acquired using an Olympus IX71 microscope.

#### 3.6. RNA Interference

The RNAi oligos for PPP3CC were purchased from Genepharma (Shanghai, China). The RNAi oligos sequences for PPP3CC were: siPPP3CC-1, 5'-GCCUCACAGUGUGUCUAAUdTdT-3' and siPPP3CC-2, 5'-GCUCGCUACUGCAUAUGAUdTdT-3'. The sequence of negative control was siNC, 5'-ACAGACUUCGGAGUACCUgTdT-3'.

#### 3.7. Quantitative RT-PCR

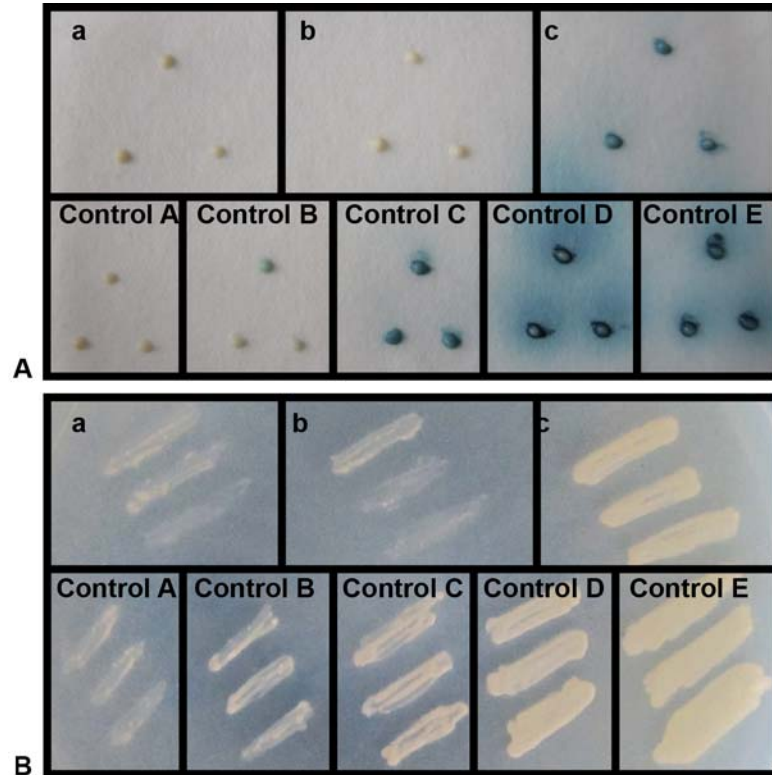
Total RNA was isolated from HEK293T cells using the Trizol reagent (Invitrogen), and cDNA was reversed-transcribed using the Reverse Transcription kit (Promega), according to the manufacturer's instructions. The primer sequences for *ITPKC* and *PPP3CC* were selected as follows: ITPKC-forward: TGGCCTTGGAGTAGAGACCG, ITPKC-reverse: CCTGTCTGGCTCCGTCTTTTG; PPP3CC-forward: ACCGCGTCATCAAAGCTGT, PPP3CC-reverse: AAAGTAAGCCGTTGGGTTGGA. PCR amplification was performed using SYBR Green PCR master mix kit, and quantitations were normalized to the level endogenous control glyceraldehyde 3-phosphate dehydrogenase.

#### 3.8. Western blot

Cell lysates and immunoprecipitates were separated by SDS-PAGE and proteins were transferred to PVDF membranes (Millipore). The membrane was blocked in PBS with 5% nonfat milk and 0.1% Tween20, and incubated with primary antibodies at room temperature for 2h, and the washed twice in PBS, followed by incubation with secondary antibody at room temperature for 1h. Immunoreactivity was visualized by using enhanced chemiluminescence (Millipore). Related antibodies in Western blot included: anti-HA antibody, anti-His antibody and anti-FLAG antibody (Sigma); anti-Myc antibody (Santa Cruz); anti-GAPDH (Kangcheng). anti-PPP3CC $\square$ PTG, anti-ITPKC (PTG) and anti-Phosphoserine (Upstate).

#### 3.9. ELISA

Double-antibody sandwich ABC-ELISA was used in this study. Plates were coated with anti human IP3 monoclonal antibody, and human IP3 in standards and samples were fixed to the antibody coat, immune complexes were formed and attached to the board by adding biotinylated antihuman IP3 antibody, and



**Figure 1.** PPP3CC interacted with ITPKC in yeast. A. LacZ reporter system assay a. pDBLeu-ITPKC + pPC86; b. pDBLeu + pPC86-PPP3CC; 3. pDBLeu-ITPKC+ pPC86-PPP3CC; Control A-E: Yeast control strain A-E. B. HIS3 reporter gene assay a. pDBLeu-ITPKC + pPC86; b. pDBLeu + pPC86-PPP3CC; 3. pDBLeu-ITPKC+ pPC86-PPP3CC; Control A-E: Yeast control strain A-E

horseradish peroxidase- conjugated Streptavidin was added and combined with biotin. Wells would change to yellow after adding the enzyme substrate OPD and the color development would be stopped by H<sub>2</sub>SO<sub>4</sub>. Read the ratio of absorptions 492 nm, and the concentration of human IP3 was proportional to the OD value, the concentration of human IP3 in samples could be calculated by drawing a standard curve.

### 3.10. Statistical analysis

The differences between groups were analyzed using Student's t-test. All analyses were performed using SPSS 13.0 software (Chicago, IL). Results were considered to be statistically significant if p<0.05.

## 4. RESULTS

### 4.1. PPP3CC, a new ITPKC-interacting protein, was identified using yeast two-hybrid screen

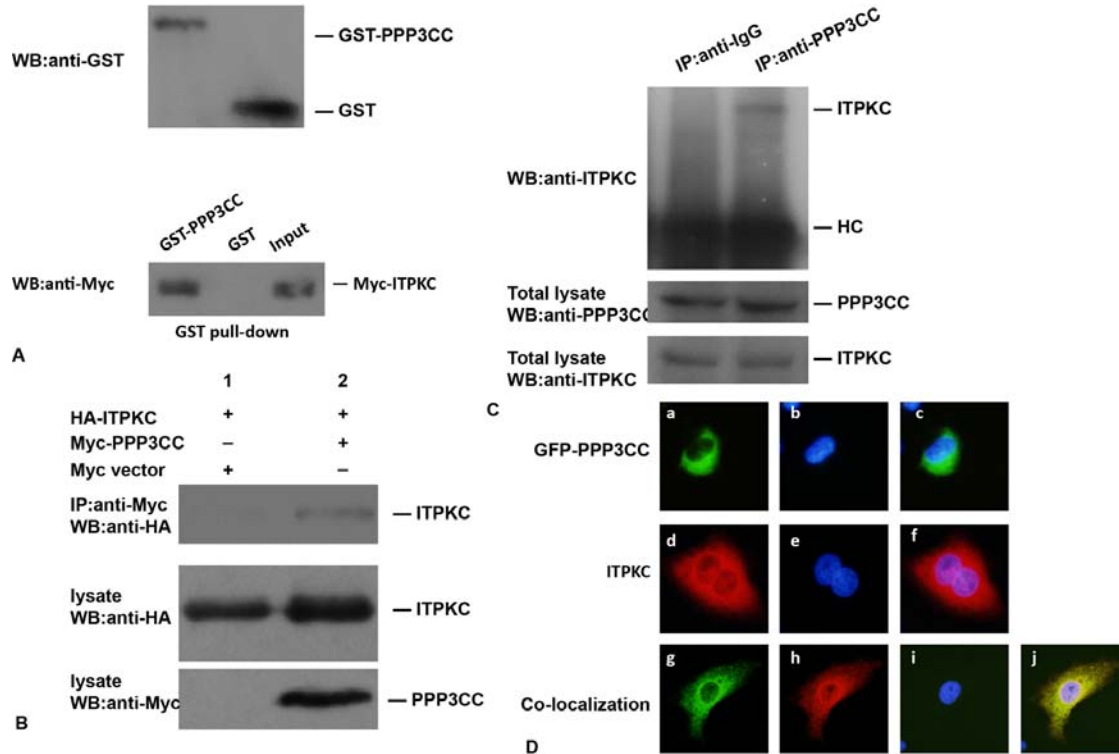
We screened *ITPKC* (GeneID: 80271) interacting protein in a human liver cDNA library by yeast two-hybrid, and a gene fragment of *PPP3CC* (GeneID: 5533) was identified after sequencing and blast analysis. To validate this interaction, full-length *PPP3CC* was cloned into the pPC86 vector and co-transformed with pDBLeu-ITPKC into yeast strain MaV203. As shown in Figure1, the interaction between full-length ITPKC and PPP3CC was confirmed in yeast.

### 4.2. ITPKC and PPP3CC interact *in vitro* and *in vivo*

To examine the ITPKC-PPP3CC protein interaction *in vitro*, the GST pull down assay was performed. As expected, the *in vitro* translated Myc-ITPKC obtained by TNT Quick Coupled Transcription/Translation System efficiently bound to GST-PPP3CC fusion protein purified from *E. coli*, but not to GST control alone (Figure 2A).

To verify the *in vivo* interaction between ITPKC and PPP3CC in mammalian cells, two eukaryotic expression plasmids, pCMV-HA-ITPKC and pCDEF-Myc-PPP3CC, were co-transfected into HEK293T cells followed by co-immunoprecipitation assay. The results showed that HA-ITPKC could be detected in the anti-Myc immunoprecipitates from the cells co-transfected with Myc-PPP3CC, but not from the cells co-transfected with an empty vector (Figure 2B), suggesting that ITPKC binds to PPP3CC in mammalian cells.

Co-immunoprecipitation experiments were also performed in untransfected cells to verify the interaction between endogenous ITPKC and PPP3CC under a physiological condition. The results showed that endogenous ITPKC could be detected in the anti-PPP3CC immunoprecipitates, but not in the non-specific antibody anti-IgG immunoprecipitates (Figure 2C), demonstrating the ITPKC-PPP3CC interaction at endogenous level.



**Figure 2.** PPP3CC interacted with ITPKC *in vitro* and *in vivo*. **A.** GST-PPP3CC interacted with Myc-ITPKC in GST pull down assay. Top panel: Recombinant GST-PPP3CC and GST protein were purified from *E.coli*. Bottom panel: GST-PPP3CC, but not GST, could bind to Myc-ITPKC obtained from *in vitro* translation system. **B.** PPP3CC interacted with ITPKC in HEK293T cells. HA-ITPKC was detected in the anti-Myc immunoprecipitates from the cells co-transfected with Myc-PPP3CC and HA-ITPKC, but not from the cells co-transfected with Myc Vector and HA-ITPKC. **C.** PPP3CC interacted with ITPKC at cellular endogenous level. ITPKC was detected in the anti-PPP3CC immunoprecipitates from cells, but in anti-IgG immunoprecipitates. **D.** Co-localization of PPP3CC and ITPKC in HeLa cells. Nuclei were stained with Hoechst 33342. a-c: PPP3CC was localized in the cytoplasm; d-f: ITPKC was localized in the nucleus and cytoplasm; g-j: PPP3CC and ITPKC were co-localization in the cytoplasm.

#### 4.3. Subcellular localization assay of ITPKC and PPP3CC

To investigate the subcellular localization of ITPKC and PPP3CC, the HeLa cells transfected with GFP-labeled PPP3CC were subjected to immunostaining for ITPKC detection by fluorescent microscopy. Our results showed that GFP-PPP3CC mainly localized in the cytoplasm, while ITPKC was distributed in both cytoplasm and nucleus (Figure 2D). Importantly, both ITPKC and PPP3CC co-localized in the cytoplasm (Figure 2D), suggesting they may function together in the cells.

#### 4.4. PPP3CC promotes the expression of ITPKC

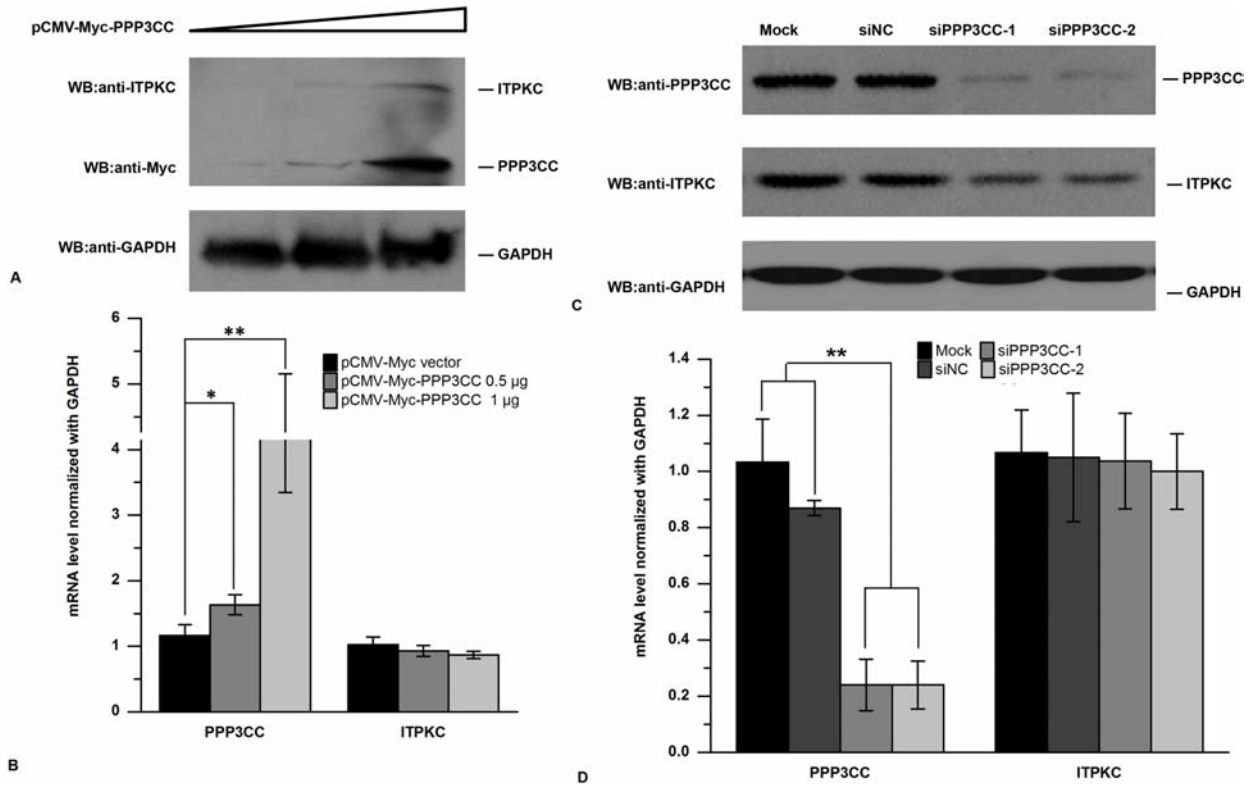
We next sought to examine the biological significance of the ITPKC-PPP3CC interaction. In an attempt to monitor the influence of PPP3CC on ITPKC protein level, we found that the expression level of ITPKC was gradually enhanced along with the increased amount of Myc-PPP3CC transfected into the cells (Figure 3A). Consistently, when the expression of PPP3CC was interfered via siRNA, the protein level of ITPKC was decreased (Figure 2C). Importantly, in neither case, the change of PPP3CC expression affected the transcriptional of ITPKC (Figure 3B and 3D), suggesting that PPP3CC positively regulates ITPKC at the posttranslational level.

To further investigate this phenotype, cells were treated with cycloheximide (CHX) to prevent protein translation process and the effect of PPP3CC on the half-life of ITPKC protein was evaluated by Western blotting. In the cells transfected with empty vectors, ITPKC was gradually reduced within two hours after CHX treatment, while in the cells transfected with PPP3CC the reduction of ITPKC was significantly diminished (Figure 4A). This reduction of ITPKC expression was also inhibited by the proteasome inhibitor MG132 (Figure 4A), indicating involvement of the proteasome degradation pathway. Together, our results suggest that PPP3CC stabilizes ITPKC by inhibiting the proteasome degradation of ITPKC.

#### 4.5. PPP3CC inhibits ubiquitination of ITPKC

As proteasome degradation pathway marks and recognizes the target proteins by ubiquitination, we next tested whether PPP3CC could influence the ubiquitination status of ITPKC. HEK293T cells were co-transfected with Flag-ITPKC, HA-tagged ubiquitin (HA-Ub), and Myc-PPP3CC or Myc-Vector control. After MG132 treatment, anti-FLAG immunoprecipitates were subjected to Western blotting using an anti-HA antibody to detect a ladder of

### PPP3CC feedback regulates IP3-Ca<sup>2+</sup> pathway



**Figure 3.** PPP3CC increased the expression level of ITPKC at protein level. A. Over-expression of PPP3CC could increase the protein expression level of ITPKC. pCMV-Myc-PPP3CC was transfected into HEK293T cells and the expression levels of PPP3CC and ITPKC were detected by Western Blot. B. Over-expression of PPP3CC did not affect the amount of mRNA quantity of ITPKC. Real-time PCR was performed to measure the variance of mRNA quantity of PPP3CC and ITPKC in HEK293T cells transfected with pCMV-Myc-PPP3CC, the mRNA level of PPP3CC was significantly up-regulated (\* represents  $p < 0.05$ , \*\* represents  $p < 0.01$ ), while the mRNA level of ITPKC did not change significantly. C. The expression level of ITPKC decreased when PPP3CC RNAi knockdown reduced the expression of PPP3CC. siRNAs of PPP3CC was transfected into HEK293T cells and the expression levels of PPP3CC and ITPKC were detected by Western Blot. D. Down-regulation of PPP3CC by RNAi did not affect ITPKC on the mRNA level. Significant decrease of mRNA quantity of PPP3CC (\*\* represents  $p < 0.01$ ) was detected by Real-time PCR after PPP3CC knockdown by RNAi, but there was no significant difference of mRNA quantity of ITPKC.

bands that represent different poly-ubiquitinated forms of ITPKC. The amount of ITPKC conjugated with HA-Ubs was remarkably reduced when Myc-PPP3CC was co-transfected compared to the Myc-Vector control alone (Figure 4B), indicating that PPP3CC inhibits the ubiquitination of ITPKC, and thus to prevent its proteasome degradation.

#### 4.6. PPP3CC reduces the phosphorylation level of ITPKC, preventing its entry into the ubiquitin-proteasome pathway

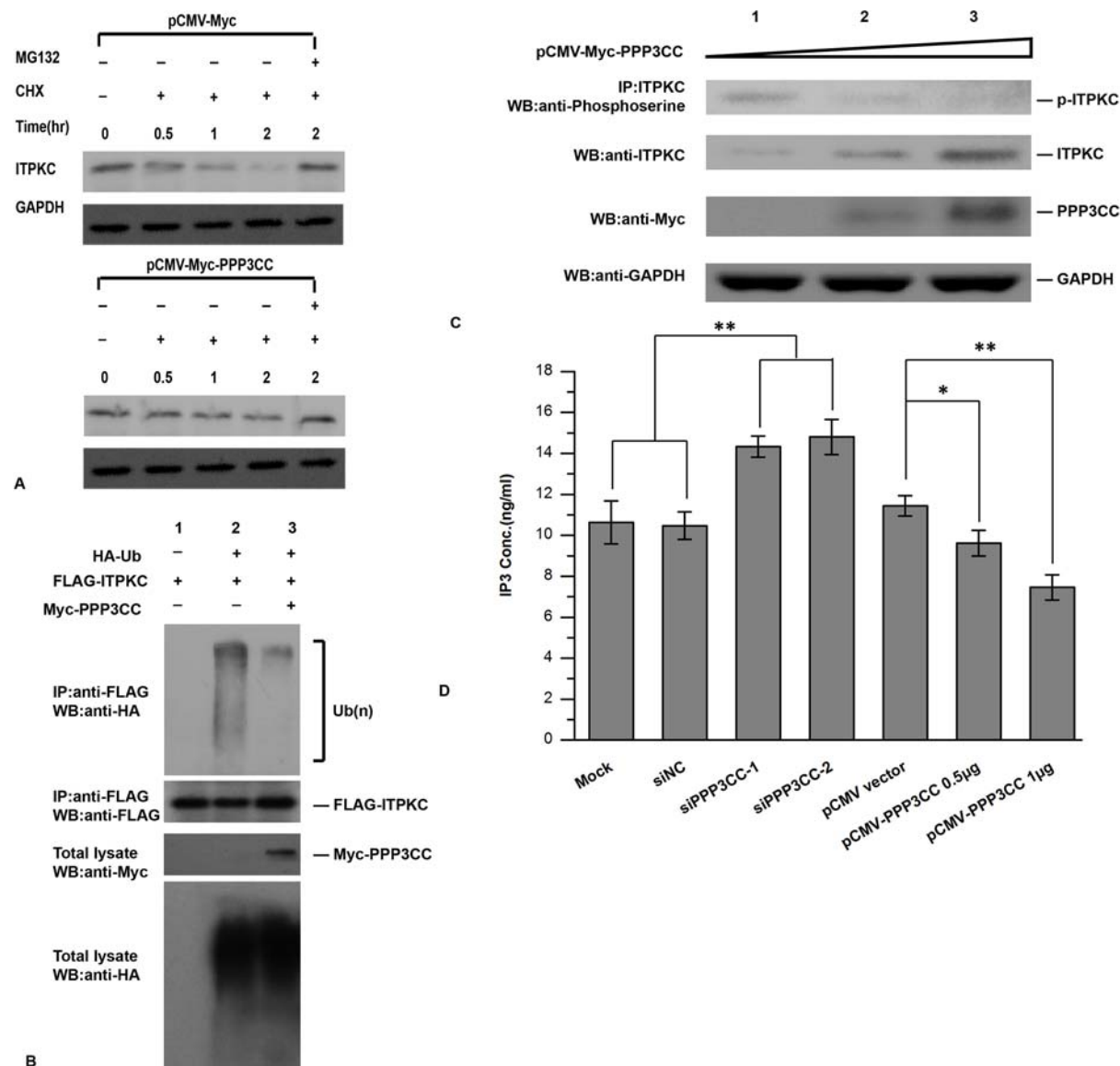
Since phosphorylation is a signal and/or prerequisite of ubiquitination for many proteins (17), we checked the effect of PPP3CC, which is a phosphatase, on the phosphorylation status of ITPKC. The level of ITPKC and p-ITPKC were measured with specific antibodies in cells transfected with different doses of Myc-PPP3CC. The results showed an increase of ITPKC and a decrease of p-ITPKC with increasing amount of PPP3CC (Figure 4C), suggesting that PPP3CC inhibits the phosphorylation of ITPKC, presumably through its phosphatase activity.

#### 4.7. PPP3CC may negatively regulate the expression level of IP3 via ITPKC

ITPKC can negatively regulate intracellular Ca<sup>2+</sup> concentration and inhibit the activation of calcineurin via phosphorylating IP3 to IP4 (18). As PPP3CC inhibits the degradation of ITPKC, we speculated that PPP3CC may indirectly reduce the amount of intracellular IP3 via ITPKC. To test this idea, Myc-PPP3CC or PPP3CC siRNAs were transfected into the cells to up-regulate or down-regulate the expression of PPP3CC, respectively, and the IP3 level in the cytoplasm was measured by ELISA. The results showed a decrease of IP3 in the cytoplasm with up-regulation of PPP3CC and an increase of IP3 with down-regulation of PPP3CC (Figure 4D), demonstrating a role of PPP3CC in negatively controlling the cytoplasmic level of IP3 likely through stabilizing ITPKC. Therefore, PPP3CC may serve as a novel negative regulator in the IP3-Ca<sup>2+</sup> signal pathway.

### 5. DISCUSSION

Inositol 1,4,5-trisphosphate (IP3) is an important secondary messenger that transduce signals from cell

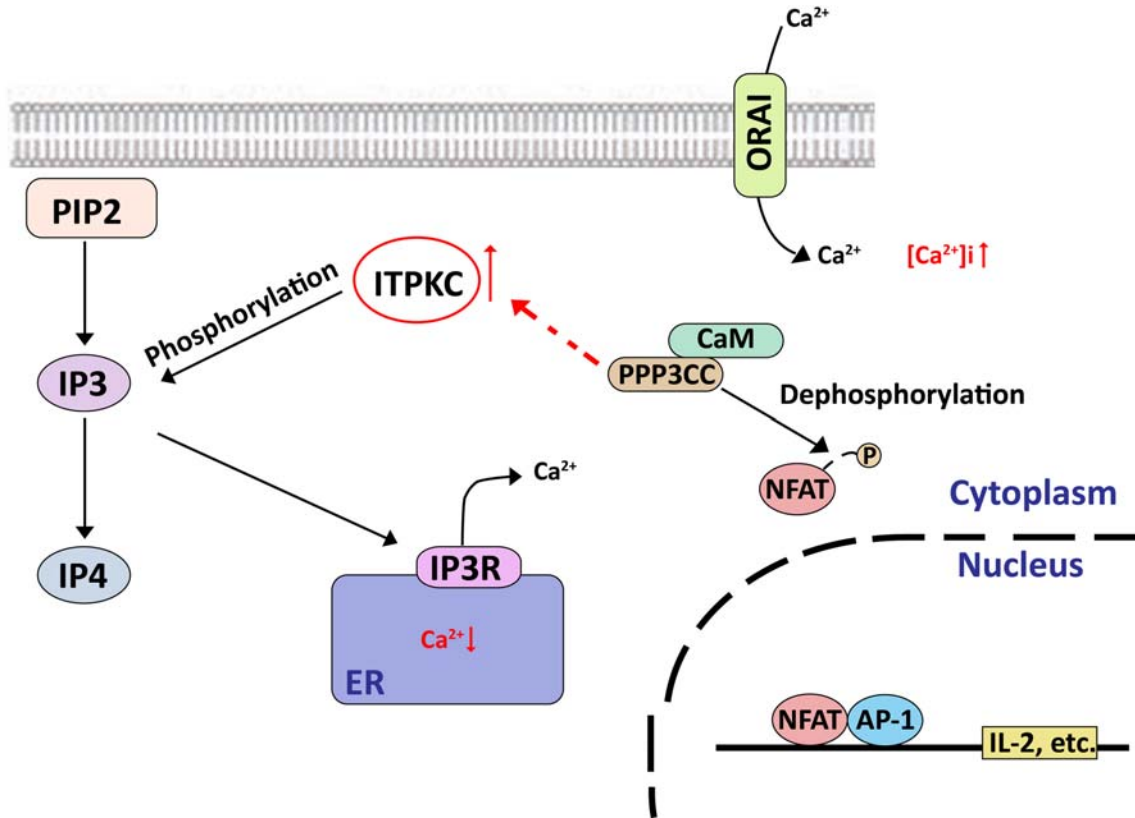


**Figure 4.** PPP3CC regulates the activation of the IP3-Ca<sup>2+</sup> pathway via stabilizing the expression of ITPKC. **A.** PPP3CC extended the half-life of ITPKC. Cells were transfected with pCMV-Myc vector or pCMV-Myc-PPP3CC respectively and treated with cycloheximide (CHX) and MG132 for indicated times. Western blot was performed to investigate the levels of ITPKC, GAPDH was detected as a loading control. **B.** PPP3CC inhibited the ubiquitination of ITPKC. HEK293T cells were co-transfected with Myc-PPP3CC, FLAG-ITPKC and HA-Ubiquitin (HA-Ub) and treated with MG132 as indicated. FLAG-ITPKC conjugated HA-Ubs were detected by Western blot in the anti-FLAG immunoprecipitates. **C.** PPP3CC inhibited the phosphorylation of ITPKC. HEK293T cells were transfected with gradually increased amount of Myc-PPP3CC as indicated. Phosphorylation of ITPKC was detected by Western blot in the anti-ITPKC immunoprecipitates. **D.** PPP3CC reduced the amount of IP3 in the cells. Myc-PPP3CC and siRNAs were transfected respectively into cells, and the amounts of IP3 were detected by ELISA.

surface receptor (19). ITPKC phosphorylates IP3 to IP4 to inhibit the activation of the downstream calcium signaling pathway. When the expression of ITPKC decreased, IP3 molecules would be accumulated and activate the IP3-Ca<sup>2+</sup> pathway in cells, subsequently activate calmodulin-dependent phosphatases, then NFAT in the cytoplasm would be dephosphorylated and translocated into nuclear to initiate the expression of downstream genes. The

abnormality of the above process might cause immune overreaction and Kawasaki disease (9, 13).

PPP3CC is a member of calcineurin catalytic subunits previously documented as a positive regulator in the NFAT signaling pathway, which catalyzes the dephosphorylation of NFAT and promotes its nuclear translocation to activate the expression of inflammation-



**Figure 5.** PPP3CC as a candidate negative regulator in IP3-Ca<sup>2+</sup>/NFAT pathway by stabilized the expression of ITPKC.

related downstream genes. PPP3CC is known to be a downstream player of ITPKC in IP3-Ca<sup>2+</sup>/NFAT signaling pathway, as ITPKC negatively regulates PPP3CC through regulation of intracellular Ca<sup>2+</sup> signal. If the expression of intracellular ITPKC is reduced, PPP3CC will be over-activated by Ca<sup>2+</sup> signal, thus lead to symptoms of over activation of immunity such as Kawasaki disease.

Strikingly, our results showed that PPP3CC stabilizes the expression of ITPKC by reducing the phosphorylation level of ITPKC, and thus preventing its entry into ubiquitin-proteasome degradation pathway. It is well-known that phosphorylation of serine and/or threonine enables the ubiquitin system to identify the phosphorylated substrate and to tag it with ubiquitin to initiate protein degradation (17). This mechanism has played an important role in the regulation of cyclins (20) and the NF-kappaB signaling pathway (21). Here, we discovered that through regulating the phosphorylation level of ITPKC, PPP3CC feedback modulated ITPKC level and balanced IP3-Ca<sup>2+</sup>/NFAT signaling pathway to prevent over-activation of NFAT pathway. It appears that the interplay between PPP3CC and ITPKC is an important dual-control mechanism for IP3-Ca<sup>2+</sup>-NFAT pathway. On one hand, reduction of ITPKC activates PPP3CC to dephosphorylates NFAT to promote IP3-Ca<sup>2+</sup>-NFAT pathway; on the other hand, in the case of over-activation of Ca<sup>2+</sup> signal, PPP3CC can play an inhibitory role through stabilizing ITPKC (Figure 5). The transduction mechanism of the Ca<sup>2+</sup> signal

has been studied in depth in the previous studies, and the activation mechanism of this signal of Calmodulin/Calcineurin is recognized for all (22, 23). However, little has been reported for the feedback regulation in this pathway. The new mechanism we found in this study will be of great help for us to understand the complexity of calcium signaling pathway.

As abundant evidence suggests that alteration of calcium signaling is involved in etiology of schizophrenia (24), and the genetic polymorphism of PPP3CC has also been shown to be associated with schizophrenia (15, 25-27), our discovery of PPP3CC's novel role in calcium signaling will generate new ventures for the mechanistic understanding of schizophrenia (28, 29). Both *ITPKC* and *PPP3CC* are susceptibility genes of disease caused by dysfunction of calcium signaling pathway. The ITPKC-PPP3CC interaction described here constitutes an IP3-Ca<sup>2+</sup> signaling feedback loop that makes important supplementary to the classic calcium signaling pathway, and provides a new theoretical basis and direction to overcome Kawasaki disease and schizophrenia.

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