



# Genome-Wide Bimolecular Fluorescence Complementation-Based Proteomic Analysis of *Toxoplasma gondii* ROP18's Human Interactome Shows Its Key Role in Regulation of Cell Immunity and Apoptosis

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*Toxoplasma gondii* rhoptry protein ROP18 (TgROP18) is a key virulence factor secreted into the host cell during invasion, where it modulates the host cell response by interacting with its host targets. However, only a few TgROP18 targets have been identified. In this study, we applied a high-throughput protein-protein interaction (PPI) screening in human cells using bimolecular fluorescence complementation (BiFC) to identify the targets of Type I strain ROP18 (ROP18<sub>I</sub>) and Type II strain ROP18 (ROP18<sub>II</sub>). From a pool of more than 18,000 human proteins, 492 and 141 proteins were identified as the targets of ROP18<sub>I</sub> and ROP18<sub>II</sub>, respectively. Gene ontology, search tool for the retrieval of interacting genes/proteins PPI network, and Ingenuity pathway analyses revealed that the majority of these proteins were associated with immune response and apoptosis. This indicates a key role of TgROP18 in manipulating host's immunity and cell apoptosis, which might contribute to the immune escape and successful parasitism of the parasite. Among the proteins identified, the immunity-related proteins N-myc and STAT interactor, IL20RB, IL21, ubiquitin C, and vimentin and the apoptosis-related protein P2RX1 were further verified as ROP18<sub>I</sub> targets by sensitized emission-fluorescence resonance energy transfer (SE-FRET) and co-immunoprecipitation. Our study substantially contributes to the current limited knowledge on human targets of TgROP18 and provides a novel tool to investigate the function of parasite effectors in human cells.

**Keywords:** *Toxoplasma gondii*, ROP18, human interactome, bimolecular fluorescence complementation, genome-wide

## INTRODUCTION

*Toxoplasma gondii* is an obligate intracellular protozoon that causes zoonotic toxoplasmosis. It is estimated that one third of the world's population is chronically infected with this parasite (1). *T. gondii* belongs to the phylum Apicomplexa, characterized by the presence of an apical complex containing secretory organelles, including rhoptries, micronemes, and dense granules (2). Rhoptry

discharges a family of proteins termed rhopty proteins (ROPs) that are of importance for host cell invasion, intracellular survival, and interference with host functions (3, 4).

*T. gondii* isolates collected from North America and Europe primarily fall into one of the three distinct clonal lineages, types I, II, and III (5), which present a number of different phenotypes, such as growth, migration, and transmigration (6). The best characterized phenotype is their virulence in laboratory mice (7, 8): Type I strains exhibit acute lethal virulence [lethal dose (LD<sub>100</sub>) ≈ 1], whereas types II and III strains are much less virulent [median LD<sub>50</sub> ≥ 10<sup>5</sup>] (9, 10). According to previous forward genetic mapping studies, in which Types I, II, or III were intercrossed to identify the virulence determinant genes, the highly polymorphic *rop18* gene was identified as a key virulence determinant (11, 12). TgROP18 is a serine/threonine kinase secreted from the rhopty into the parasitophorous vacuole membrane (PVM) and host cytosol during parasite invasion (13), of which the Type I strain (ROP18<sub>I</sub>) (RH strain, GenBank accession NO: AF054817.1) and the Type II strain (ROP18<sub>II</sub>) (ME49, GenBank accession NO: XP\_002367757.1) are different at 28 amino acid sites.

In murine cells, ROP18<sub>I</sub> can target and inactivate the immunity-related GTPases (IRGs) Irga6 and Irgb6 by phosphorylating a critical threonine residue in the switch loop 1 of the IRGs, thereby disrupting their accumulation on the PVM and protecting the parasites from destruction (14, 15). Although the precise molecular functions of TgROP18 in human cells remain obscure, it is known that it regulates parasite's multiplication in human cells and host cell apoptosis. It has been reported that a Type III strain (CEP) expressing ROP18<sub>I</sub> showed a dramatic increase in replication rate in human foreskin fibroblasts (HFFs) in comparison to the wild-type CEP strain without TgROP18 expression (13). It has also been shown that ROP18<sub>I</sub> inhibits cell apoptosis *via* the mitochondrial apoptosis pathway in human embryonic kidney 293 T cells (16). TgROP18 exerts its regulation on some important host cell signaling by interacting with its host targets. For instance, ROP18<sub>I</sub> phosphorylates and mediates the degradation of the host endoplasmic reticulum (c)-bound transcription factor ATF6β, which is expressed in both human and murine cells, resulting in compromised CD8<sup>+</sup> T cell-mediated host defense against *T. gondii* infection (17). ROP18<sub>I</sub> has also been shown to associate with p65, a member of the human NF-κB family of transcription factors, and targets this protein for ubiquitin-dependent degradation to suppress the human NF-κB pathway (18). Despite the important roles of the virulence factor TgROP18 in disrupting host cell functions and preserving survival of parasites in human cells, only a few binding partners of ROP18<sub>I</sub> have been determined, and the complex regulatory network of protein–protein interactions (PPIs) between ROP18<sub>I</sub> and host cell proteins remains to be elucidated. Moreover, very little is known regarding the host targets of ROP18<sub>II</sub>, even though it is functionally expressed in Type II strains and is capable of conferring virulence to a Type III strain (11, 19).

A previous study has identified eight TgROP18-interacting proteins with a yeast two-hybrid (YTH) system (20). However, YTH generates a high occurrence of false positives and requires

that the interacting proteins accumulate in the yeast nucleus (21). More recently, by using a protein array approach, Yang et al. have identified 68 substrates of the TgROP18 kinase, and four of them have been validated as the host targets (22). Although protein array is useful for comprehensive screens of protein functions, it requires pure functional proteins that are difficult to obtain because of the difficulties in expressing the proteins in a soluble form with correct folding (23). The bimolecular fluorescence complementation (BiFC) technique has been proven to be a useful and efficient tool to study PPIs. The BiFC assay is based on the principle that two non-fluorescent fragments [e.g., amino-yellow fluorescence protein, NYFP, or carboxyl-yellow fluorescence protein, C-terminal fragment of YFP (CYFP)] of a fluorescent reporter protein (e.g., yellow fluorescence protein, YFP) can refold together and reconstitute the functional fluorescent entity when they are in close proximity, for example by fusing to a pair of interacting proteins (Figure 1A) (24). Thus, the fluorescence intensity is proportional to the amount of formed dimer and can be detected by microscopy or flow cytometry (25). Here, we applied a high-throughput PPI screening based on BiFC (HT-BiFC) combined with a Gateway cloning system (26) to identify the potential TgROP18 (ROP18<sub>I</sub> and ROP18<sub>II</sub>) interaction partners within a human ORFome library containing more than 18,000 human cDNA clones (27). This screening helped us gain insights into the biological functions of TgROP18 in human cells.

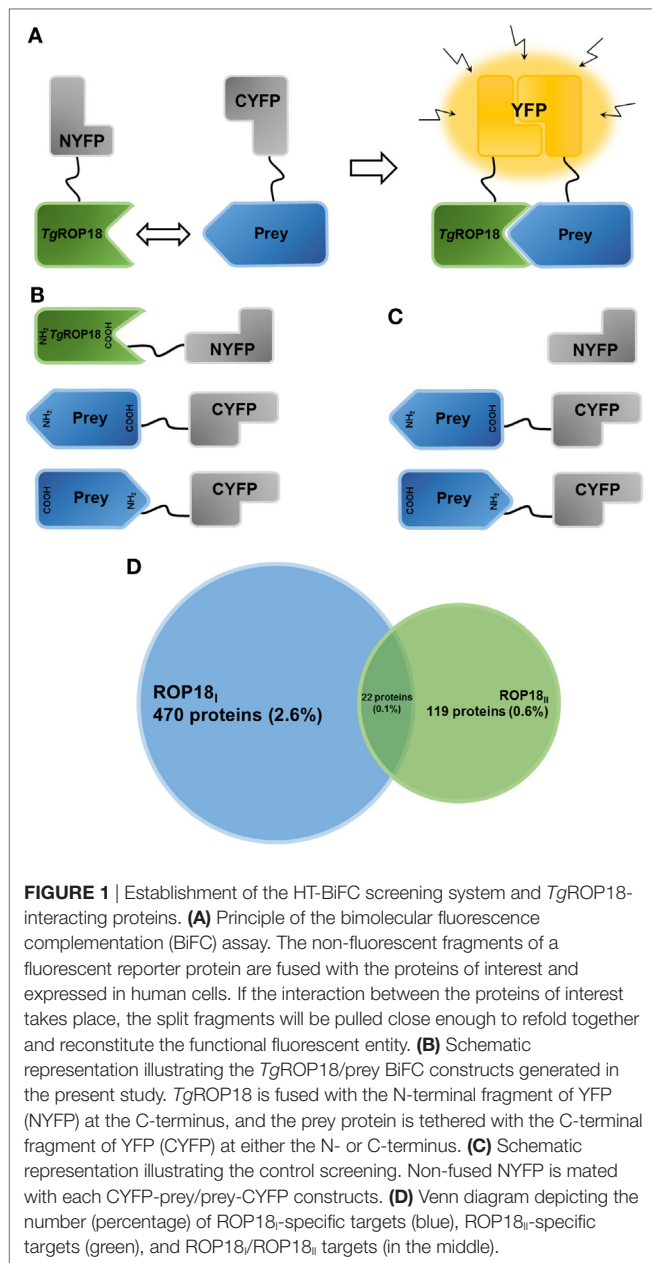
## MATERIALS AND METHODS

### Parasites and Cell Lines

The RH and PRU strains of *T. gondii* were maintained by serial passage in HFFs, as described previously (28). The HFFs (#ATCC SCRC-1041), Phoenix (#ATCC CRL-3213), and COS-7 (#ATCC CRL-1651) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The HTC75 cell line was kindly provided by Professor Wenbin Ma (Sun Yat-Sen University, Guangzhou, China). Parasites and cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, #11995065) supplemented with 10% fetal bovine serum (Gibco, #16000044) and 1% penicillin/streptomycin (Gibco, #15070063) at 37°C in a 5% CO<sub>2</sub> incubator.

### Antibodies

Anti-NMI rabbit monoclonal antibody (#183724) was obtained from Abcam (Cambridge, MA, USA). Anti-FLAG mouse monoclonal antibody (#AE005) was obtained from Abclonal (Woburn, MA, USA). Anti-HA rabbit monoclonal (#3724) and anti-β-Actin rabbit monoclonal (#4970) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Normal rabbit control IgG (#AB-105-C) was obtained from R&D Systems (Minneapolis, MN, USA). Anti-P2RX1 goat polyclonal (#sc-31491) and normal goat IgG (#sc-2028) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Normal mouse IgG (#12-371) was obtained from Sigma-Aldrich (Billerica, MA, USA). Mono- and polyubiquitinated conjugates monoclonal (FK2) antibody (#BML-PW8810) was obtained from Enzo Life Sciences (Farmingdale, NY, USA).



**FIGURE 1** | Establishment of the HT-BiFC screening system and *TgROP18*-interacting proteins. **(A)** Principle of the bimolecular fluorescence complementation (BiFC) assay. The non-fluorescent fragments of a fluorescent reporter protein are fused with the proteins of interest and expressed in human cells. If the interaction between the proteins of interest takes place, the split fragments will be pulled close enough to refold together and reconstitute the functional fluorescent entity. **(B)** Schematic representation illustrating the *TgROP18*/prey BiFC constructs generated in the present study. *TgROP18* is fused with the N-terminal fragment of YFP (NYFP) at the C-terminus, and the prey protein is tethered to the C-terminal fragment of YFP (CYFP) at either the N- or C-terminus. **(C)** Schematic representation illustrating the control screening. Non-fused NYFP is mated with each CYFP-prey/prey-CYFP constructs. **(D)** Venn diagram depicting the number (percentage) of ROP18<sub>I</sub>-specific targets (blue), ROP18<sub>II</sub>-specific targets (green), and ROP18<sub>I</sub>/ROP18<sub>II</sub> targets (in the middle).

## Plasmid Construction

Total RNA of *T. gondii* RH and PRU tachyzoites was extracted using the RNeasy Plus Mini Kit (#74034, Qiagen, Germantown, MD, USA) following manufacturer's instructions. The cDNA fragments of ROP18<sub>I</sub> (ToxoDB #TGGT1\_205250) and ROP18<sub>II</sub> (ToxoDB #TGME49\_205250) were amplified by RT-PCR from the total RNA of the RH and PRU tachyzoites with the forward primer 5'-ATAGCGGCCGCAATGTTTTCGGTACAGCG-3' and the reverse primer 5'-GGCGGCCCTTCTGTGTGGAGATG-3'. The cDNAs of ROP18<sub>I</sub> and ROP18<sub>II</sub> were then fused with the N-terminal fragment (residues 1–155) of yellow fluorescent protein (NYFP) at the C-terminus to construct the bait vectors, pBabe-CMV-ROP18<sub>I</sub>-NYFP-neo and pBabe-CMV-ROP18<sub>II</sub>-NYFP-neo,

respectively (**Figure 1B**). The cDNAs of N-myc and STAT interactor (NMI), interleukin 20 receptor- $\beta$  (IL20RB), purinergic receptor P2X1 (P2RX1), interleukin 21 (IL21), ubiquitin C (UBC), and vimentin were individually amplified by PCR from the human ORFeome v3.1 (Open Biosystems) and subcloned into pcDNA3.1 for eukaryotic expression, or into pEYFP-C1 for expression fused with enhanced yellow fluorescent protein. In addition, ROP18<sub>I</sub> and ROP18<sub>II</sub> cDNAs were, respectively, subcloned into pcDNA3.1 for eukaryotic expression, and into pECFP-N1 for expression fused with enhanced cyan fluorescent protein. All constructs were verified by DNA sequencing.

## HT-BiFC Assay

The HT-BiFC screening was conducted by Longjie Biotechnology Co., Ltd. (Foshan, Guangdong, China). Bait vectors were transfected into the packaging cell lines, Phoenix cells, to generate the retrovirus, and the harvested retroviruses were used to infect HTC75 cells. Stable bait cell lines expressing ROP18<sub>I</sub>-NYFP or ROP18<sub>II</sub>-NYFP were obtained after 10 days of selection with 300  $\mu$ g/mL G418. Meanwhile, a pool of prey vectors were constructed from the human ORFeome v7.1 library, using the Gateway recombination system. At the end of the process, 17,076 colonies with a coverage of 93% of all human ORFs were successfully obtained (27). The prey collections were tethered to the C-terminal fragment (residues 156–239) of YFP (CYFP) at either the N- or C-terminus (pCL-CMV-prey-CYFP-puro and pCL-CMV-CYFP-prey-puro) (**Figure 1B**). CYFP-tagged prey retroviruses were produced as mentioned above and used to infect the stable NYFP tagged ROP18<sub>I</sub> (or ROP18<sub>II</sub>) bait cells. Two days after infection, the infected cells were subjected to 5–10 days of selection with 1  $\mu$ g/mL puromycin to obtain the stable cell lines co-expressing NYFP-tagged ROP18<sub>I</sub> (or ROP18<sub>II</sub>) and CYFP-tagged prey. All procedures were performed in 96-well plates, using the Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Brea, CA, USA). The resulting diploid cells were then harvested, and the fluorescent cells were sorted out using the LSRII flow cytometer equipped with a high-throughput sampler (BD Biosciences, San Jose, CA, USA), along with the HTC75 cells infected with only CYFP-EV retroviruses as the negative control group. The positive fluorescent cells were harvested and subjected to another round of sorting until the desired positive rate (more than 90%) was reached (Figure S1 in Supplementary Material). mRNAs of the final positive cells were extracted and reverse-transcribed into cDNA by RT-PCR amplification and were then identified through Illumina/Solexa sequencing (29).

To determine the false-positive BiFC signals resulting from the self-assembly of the two YFP fragments, a control screening was performed, in which a stable bait cell line was generated to express NYFP without fusion to ROP18<sub>I</sub> or ROP18<sub>II</sub>. The expressed NYFP was then mated with each CYFP-prey/prey-CYFP in the prey library (**Figure 1C**). The Original Total Reads of each prey was calculated through the high-throughput sequencing analysis of the whole prey library, and the NYFP Total Reads were calculated through the sequencing analysis of the positive cells obtained

from the control screening. A Bias Ratio was then defined as the tendency of the NYFP fragment to associate with the CYFP-prey/prey-CYFP, by comparing the NYFP Total Reads to the Original Total Reads for each prey. The higher the Bias Ratio, the higher risk of identifying the prey as a positive signal. The preys with a Bias Ratio of more than 1% were regarded as false-positives and discarded.

## SE-FRET Assay

The day before transfection, a total of  $1 \times 10^5$  COS-7 cells were seeded in each well of a 12-well plate with 1 mL DMEM growth medium (no antibiotics). When the cells were about 60% to 80% confluent, 1  $\mu$ g of pEYFPC1-NMI (pEYFPC1-IL20RB, pEYFPC1-P2RX1, pEYFPC1-IL21, pEYFPC1-UBC, or pEYFPC1-vimentin) and/or 1  $\mu$ g of pECFPN1-ROP18<sub>i</sub> plasmids were transfected into COS-7 cells for the experimental groups, using Lipofectamine 2000 transfection reagent (#11668-019, Invitrogen, Waltham, MA, USA). For the negative control group, pECFPN1 and pEYFPC1 empty vectors were transfected into the cells, while for the positive control group, pECFPN1-EYFP was transfected into the cells. At 6 h post-transfection, the medium was replaced with fresh complete growth medium.

For the SE-FRET assay, prior to the testing of co-transfection samples, the images of the donor (CFP-ROP18<sub>i</sub> only) and acceptor (YFP-prey only) channels were collected to determine the spectral bleed-through. The images of the donor, acceptor, and FRET channels were simultaneously collected for selection of the region of interest during detection of the samples co-transfected with CFP-ROP18<sub>i</sub> and YFP-prey. The adjusted fluorescence density was obtained by subtraction of the background light density from the fluorescence density of the protein signal. The fluorescence signal, FRET efficiency, and distance between donor and acceptor were analyzed and calculated using the Olympus FluoView FV1000 viewer software (Olympus, Tokyo, Japan).

## Co-immunoprecipitation (Co-IP) Assay

COS-7 cells overexpressing ROP18<sub>i</sub> and/or NMI (IL20RB, P2RX1, IL21, or vimentin) were prepared as mentioned previously in the FRET assay. Cell extracts were prepared by lysing the cells in cell lysis buffer (#P0013, Beyotime, Shanghai, China) with 1 mM phenylmethanesulfonyl fluoride (#WB-0181, Beijing Dingguo Changsheng Biotechnology, Beijing, China). Cell lysates were incubated with the primary antibody (anti-NMI rabbit monoclonal antibody anti-HA rabbit monoclonal antibody, anti-P2RX1 goat polyclonal antibody, or anti-FLAG mouse monoclonal antibody) with gentle rotation for 1 h at 4°C. Protein A-Agarose (#sc-2001, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was then added to the immunoprecipitation reaction with incubation overnight at 4°C. The immunoprecipitates were washed four times with phosphate-buffered saline and then eluted by boiling with SDS-PAGE loading buffer (#9173, TAKARA, Kusatsu, Japan). The eluates were analyzed by western blotting with the indicated antibodies, as described previously (28). For the UBC experiment, cells were treated with 10  $\mu$ M proteasome inhibitor MG132 (#S1748, Beyotime, Shanghai, China) for 12 h before harvesting.

## Data Analysis

Each protein sequence and functional information was obtained from the UniProt Database (<http://www.uniprot.org/>). To further define the biological functions of the TgROP18 interactome, the TgROP18-interacting proteins were analyzed using DAVID Bioinformatics Resources 6.8 (30, 31) for gene ontology (GO) annotation and enrichment analysis. Pathway analyses were done using Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, [www.ingenuity.com](http://www.ingenuity.com)) by importing the Entrez GeneID of the TgROP18-interacting proteins into online servers. Additionally, a combination of the search tool for the retrieval of interacting genes/proteins (STRING) version 10.0 database (32) and Cytoscape version 3.4.0 (33) was used to explore and build the PPI network. Statistical analysis data are presented as mean  $\pm$  SD. Student's *t*-test was utilized for statistical analysis to evaluate the significant difference between different groups using IBM SPSS Statistics 20.0 (34). Statistical significance was accepted if  $p < 0.05$ .

## RESULTS

### Characterization of the TgROP18 Interactome

After multiple rounds of flow cytometric sorting, the final positive sorting rate of the HTC75 cells co-expressing ROP18<sub>i</sub>-NYFP and CYFP-tagged prey and the HTC75 cells co-expressing ROP18<sub>ii</sub>-NYFP and CYFP-tagged prey were 93.2 and 98.6%, respectively (Figure S1 in Supplementary Material). After background noises in sequencing were filtered out using a cutoff value of five in total reads, 492 ROP18<sub>i</sub> (2.88%) and 141 ROP18<sub>ii</sub> (0.83%) interacting proteins were identified, compared to control cells (Figure 1D). Tables S1 and S2 in Supplementary Material present the list of ROP18<sub>i</sub>- and ROP18<sub>ii</sub>-interacting proteins with their total reads, respectively. Based on the specificity of the interaction, we classified the interacting proteins into three groups: A. 470 ROP18<sub>i</sub>-specific targets; B. 119 ROP18<sub>ii</sub>-specific targets; and C. 22 targets for both ROP18<sub>i</sub> and ROP18<sub>ii</sub> (Figure 1D). Regarding the ROP18<sub>i</sub> specific targets, many were ribosomal proteins (e.g., RPL23, RPL11, RPL37A, RPS14, and RPS6), GTPases (e.g., SAR1B, ARL17B, and RND2), and receptors (e.g., PTPRF, FPR1, IL9R, IL20RB, and KLRD1), whereas for the ROP18<sub>ii</sub> specific targets, many were transmembrane proteins (e.g., TM4SF20, CMTM3, TMEM147, and TMBIM4) and zinc finger proteins (e.g., ZNF232, ZSCAN2, ZSCAN32, and ZNF273). Interestingly, we found that some humoral regulating factors (e.g., UTS2, CST2, and DEFB129) and some enzymes (e.g., DEGS1 and TPO) were targeted by both ROP18<sub>i</sub> and ROP18<sub>ii</sub>.

Among the ROP18<sub>i</sub>-interacting proteins, CNBP, DCTD, NUP160, and PRAC, which had been previously confirmed as ROP18<sub>i</sub>-interacting proteins by a previous human proteome array (22), were also identified in our HT-BiFC assay, indicating the reliability and quality of our results. In addition to these known interactions, 488 interactions of ROP18<sub>i</sub> with human proteins were newly defined in our study. Notably, to our knowledge, our findings provided the first report of ROP18<sub>ii</sub>-interacting proteins in human cells and specifically identified 141 ROP18<sub>ii</sub>-interacting human proteins.

## Validation of the TgROP18-Interacting Proteins

To further validate the interactions identified by the HT-BiFC assay, six ROP18<sub>i</sub>-interacting proteins (NMI, IL20RB, P2RX1, IL21, UBC, and vimentin) with a broad range of total reads (169793, 30861, 25144, 2214, 116, and 6, respectively) were selected for two independent assays, the SE-FRET assay and the Co-IP assay.

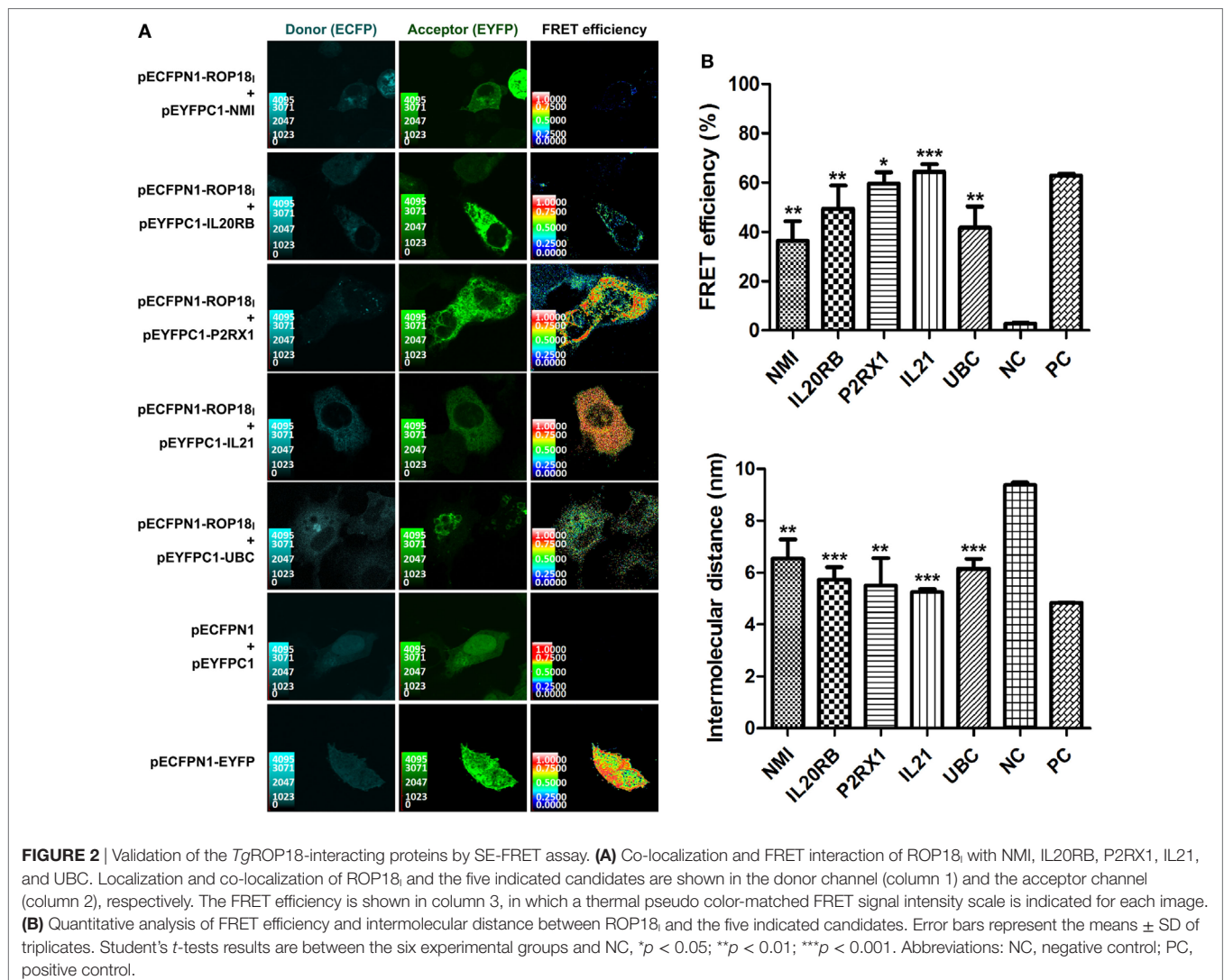
In the SE-FRET assay (Figure 2A), the donor and acceptor channels show the co-localizations of ROP18<sub>i</sub> with NMI, IL20RB, P2RX1, IL21, or UBC in the cytoplasm, suggesting the potential PPIs and their cytoplasmic localization. In addition, positive FRET signals were observed in the positive control cells and the COS-7 cells co-expressing ROP18<sub>i</sub> and NMI, IL20RB, P2RX1, IL21, or UBC (Figure 2A), yielding significantly higher FRET efficiency and less intermolecular distance than negative control cells ( $p < 0.05$ , see Figure 2B). The SE-FRET results of ROP18<sub>i</sub> and vimentin have been published recently in a study from our laboratory (35). These findings demonstrate the stable interactions

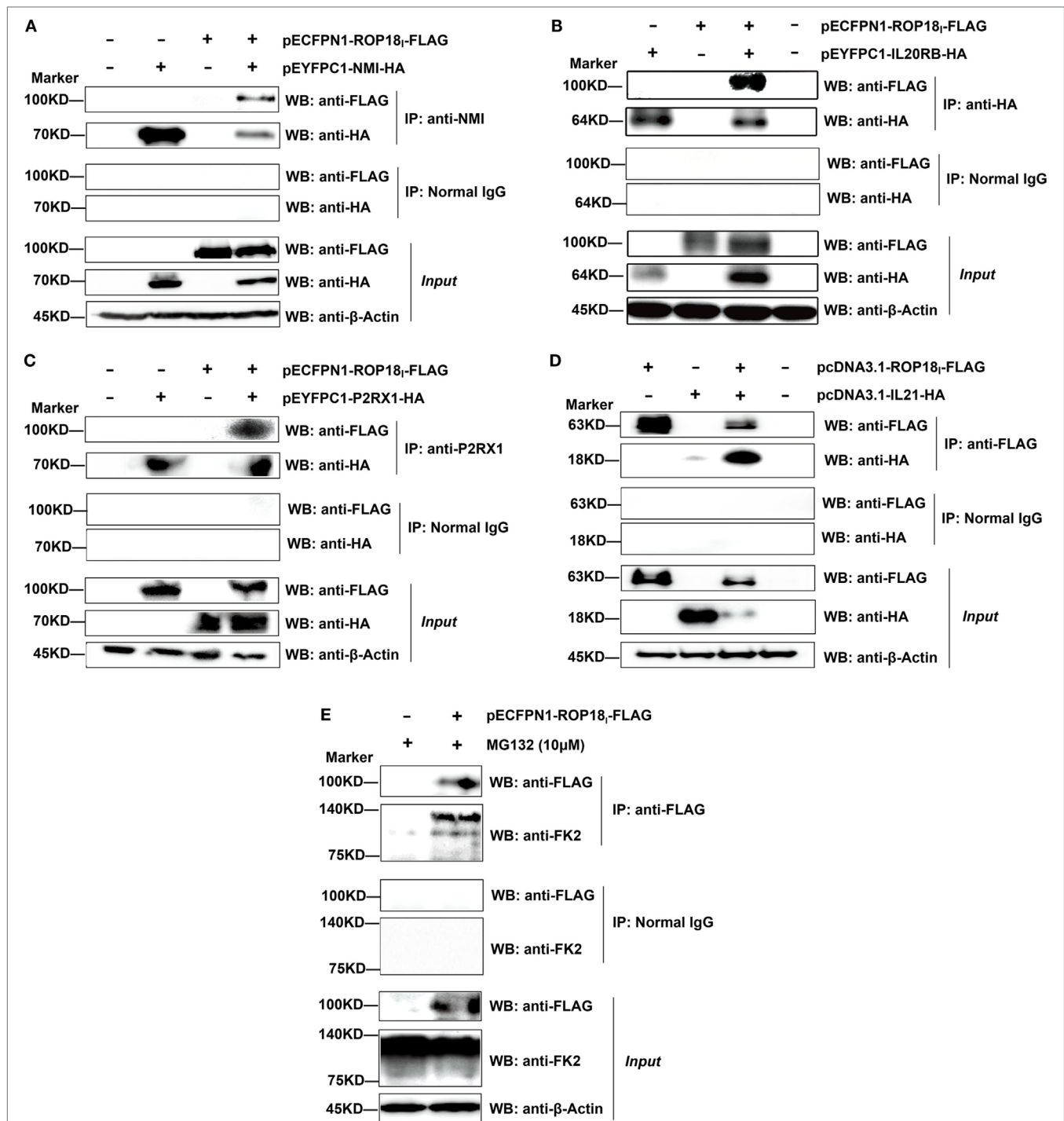
of ROP18<sub>i</sub> with NMI, IL20RB, P2RX1, IL21, UBC, and vimentin in the cytoplasm and are consistent with the HT-BiFC results, despite slight differences between the FRET efficiency values and total reads.

The interactions were also confirmed by our three replicates of Co-IP assays (Figure 3). The results show that in the dually transfected cells, ROP18<sub>i</sub> could be readily detected in the immunoprecipitates by using the specific antibodies anti-NMI, HA, P2RX1, and FLAG, but not with the control IgG. The Co-IP result of ROP18<sub>i</sub> and vimentin has been published recently in a study from our laboratory (35). These results confirmed the consistency between the two assays, suggesting the robustness and reliability of the HT-BiFC results.

## Bioinformatic Analysis of the TgROP18-Interacting Proteins

To obtain a comprehensive view of the TgROP18 interactome, we performed a GO analysis to identify significantly enriched functional terms of TgROP18-interacting proteins. The top

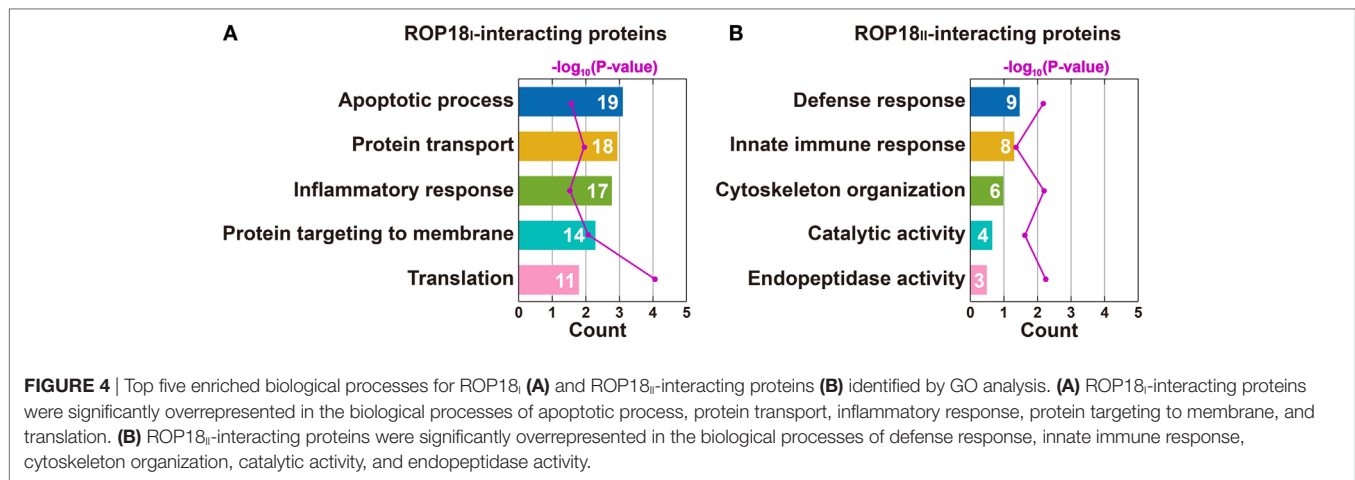




**FIGURE 3** | Validation of the TgROP18-interacting proteins by co-immunoprecipitation (Co-IP) assay. **(A–D)** Lysates of COS-7 cells co-overexpressing ROP18<sub>I</sub> and the indicated interacting proteins were immunoprecipitated with the indicated antibodies. Rabbit, mouse, or goat normal control IgG were used as negative controls. The immunoprecipitates were detected by SDS-PAGE and western blotting using the antibodies indicated. **(E)** Lysates of COS-7 cells overexpressing ROP18<sub>I</sub> in the presence of MG132 (10 μM) for 12 h were immunoprecipitated with the anti-FLAG antibody. Endogenous UBC (a smear of bands) was detected in the immunoprecipitates through western blotting with anti-FK2 antibody, which recognizes mono- and polyubiquitinated conjugates.

five enriched terms within the “Biological Process” ontology category, together with their protein counts and *p*-values, are shown in **Figure 4**. The results reveal that both ROP18<sub>I</sub> and

ROP18<sub>II</sub>-interacting proteins were significantly enriched in a variety of biological processes (*p* < 0.05). As expected, ROP18<sub>I</sub>-interacting proteins were significantly overrepresented in the



biological processes of apoptotic process ( $p = 2.7 \times 10^{-2}$ ), inflammatory response ( $p = 1.1 \times 10^{-2}$ ), and protein targeting to membrane ( $p = 8.7 \times 10^{-3}$ ), and for ROP18<sub>ii</sub>, we also added host targets to the expected biological processes, including defense response ( $p = 7.8 \times 10^{-3}$ ) and innate immune response ( $p = 4.0 \times 10^{-2}$ ). In addition to the roles of TgROP18 in the expected biological processes mentioned above, interesting roles of ROP18<sub>i</sub> in protein transport ( $p = 3.0 \times 10^{-2}$ ) and translation ( $p = 8.5 \times 10^{-5}$ ), and ROP18<sub>ii</sub> in cytoskeleton organization ( $p = 6.9 \times 10^{-3}$ ), catalytic activity ( $p = 2.2 \times 10^{-2}$ ), and endopeptidase activity ( $p = 6.1 \times 10^{-3}$ ) were also identified with great significance.

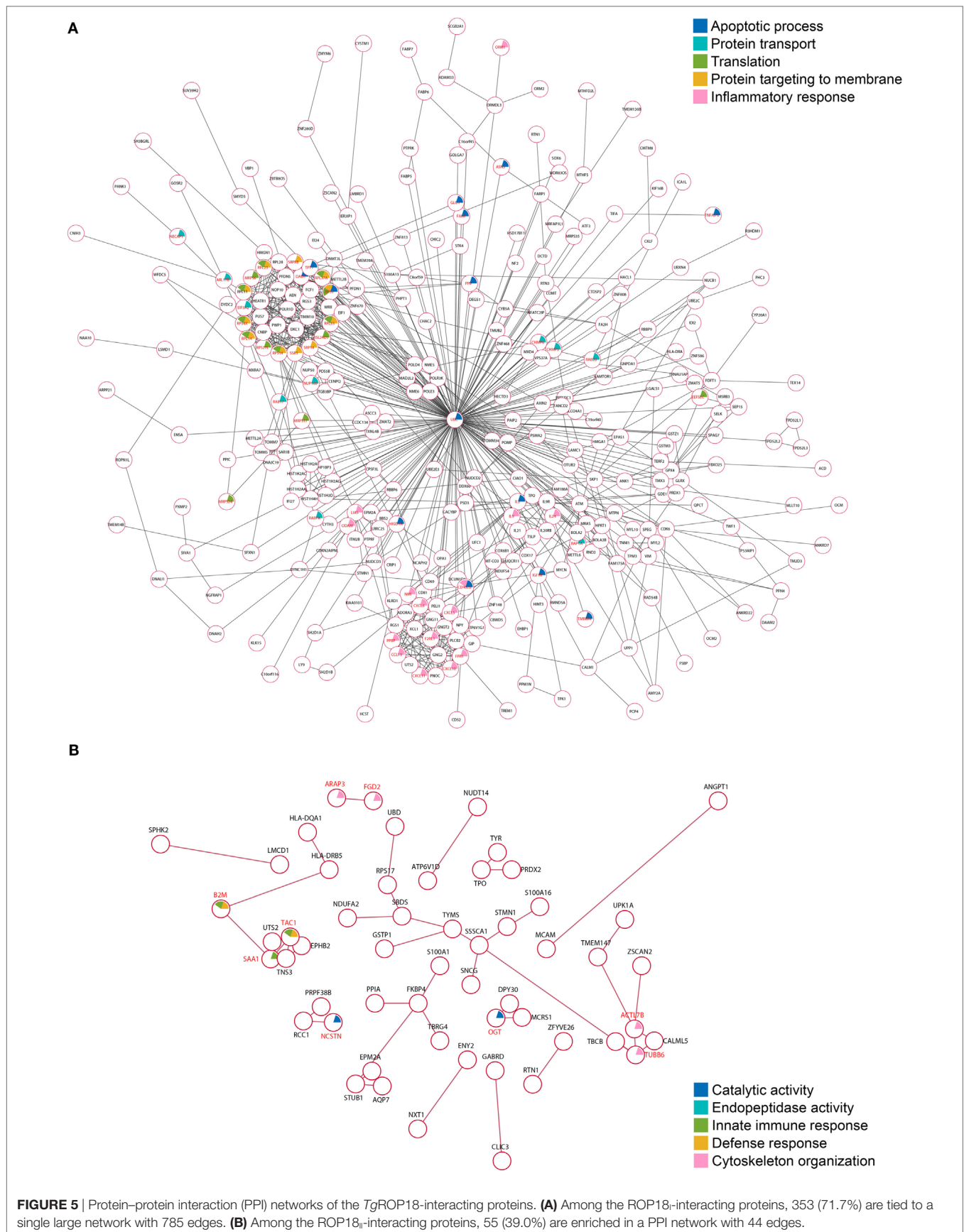
To elucidate whether the TgROP18-interacting proteins were functionally related, we conducted a deeper exploration of the PPI networks by using the STRING 10.0 database. By applying a medium confidence ( $p > 0.4$ ), 353 (71.7%) of the ROP18<sub>i</sub>-interacting proteins were tied to a single large network with a PPI enrichment  $p$ -value  $< 0.001$ ; whereas for the ROP18<sub>ii</sub>-interacting proteins, 55 (39.0%) were enriched in a large PPI network, with a PPI enrichment  $p$ -value of 0.009 (Figure 5), which indicated that each one of the two sets of interacting proteins were biologically connected as a network with a significantly greater number of interactions, rather than as a random set of proteins. As shown in Figure 5A, 785 edges (PPIs) were observed among the 353 ROP18<sub>i</sub>-interacting proteins, and seven protein–protein-interacting clusters were evident in the network, such as a cluster of ribosomal proteins containing RPS4X, RPL35, RPL23, RPL37A, and RPS6; a cluster of chemokines containing CXCL6, CXCL5, CCL19, CXCL11, and CXCL10; and a cluster of interleukins containing IL2, IL9, IL21, and IL24. Notably, UBC was observed as a main hub situated in the core of the network with 211 edges. Among the 55 ROP18<sub>ii</sub>-interacting proteins, 44 edges and four protein–protein-interacting clusters were determined by STRING analysis (Figure 5B). The cytoskeleton proteins ACTL7B, TUBB6, and TBCB were in close proximity and formed a cluster; TNS3 and UTS2 tensins were closely clustered with a tachykinin, TAC1; several functional regulators, such as TBRG4, PPIA, S100A1, and FKBP4 were tied together as a cluster; and several disease-related proteins, such as SNCG, STMN1, SSSCA1, and S100A16, were identified to be clustered.

By using the IPA database, we carried out an Ingenuity pathway analysis to further investigate the significant human signaling pathways influenced by ROP18<sub>i</sub>/ROP18<sub>ii</sub>. Among the 492 ROP18<sub>i</sub>-interacting proteins, 71 (14.4%) were mapped to 34 pathways, and 13 (9.2%) out of the 141 ROP18<sub>ii</sub>-interacting proteins were mapped to 16 pathways in total. All the involved significant pathways with their  $p$ -values and associated molecules are listed in Table S3 in Supplementary Material. The top five enriched pathways for the ROP18<sub>i</sub> and ROP18<sub>ii</sub>-interacting proteins shown in Figure 6 were closely related to cell growth, cytokine signaling, and cellular immune response. For the ROP18<sub>i</sub>-interacting proteins, MRAS was involved in the higher number of pathways, followed by ATM; whereas for the ROP18<sub>ii</sub>-interacting proteins, human leukocyte antigen (HLA)-DRB5 was involved in the higher number of pathways, followed by HLA-DQA1.

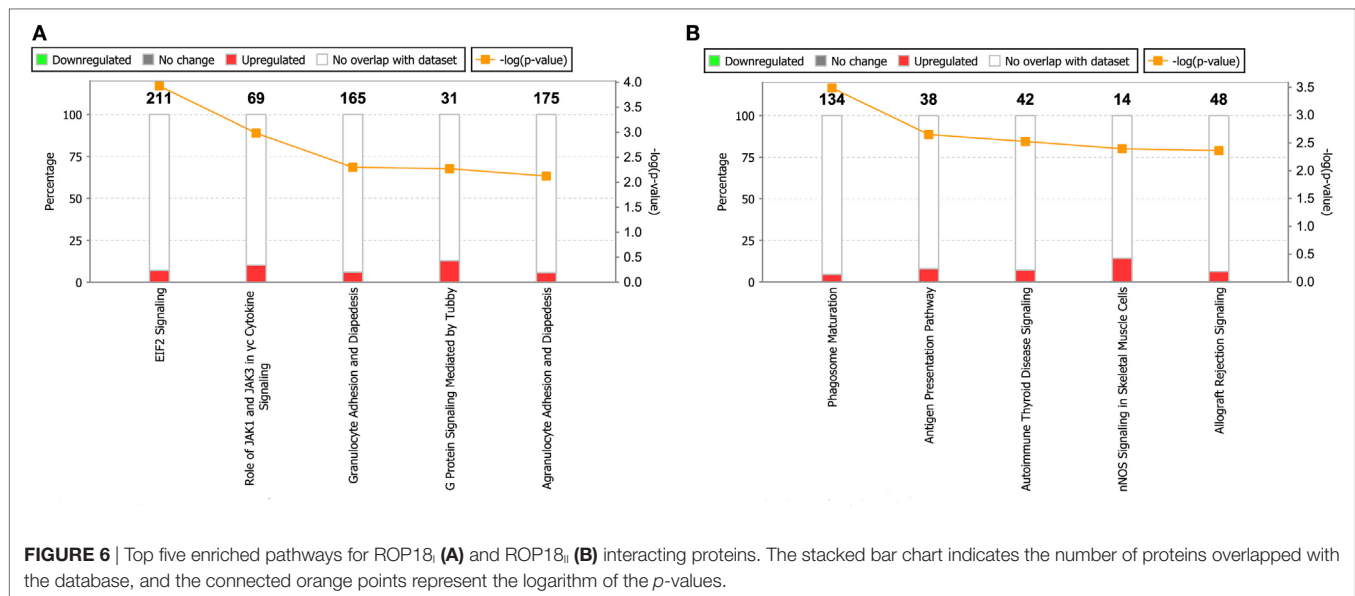
## DISCUSSION

Protein–protein interaction plays indispensable roles in structuring and regulating biological processes in all biological systems. The “protein–protein interactome” refers to the whole union of all PPIs in a particular cell or organism (36). In addition to serving as a foundation for more detailed studies on the prediction of protein functions or disease associated genes (37, 38), interactome mapping has become a critical and powerful postgenomic research tool that facilitates a better understanding of genotype-to-phenotype relationship and biological systems (39). TgROP18, which is a key virulence determinant of *T. gondii*, modulates the host cell and mediates the parasite virulence by interacting with host proteins. However, only a few host targets of ROP18<sub>i</sub> have been identified, and knowledge about the host targets of ROP18<sub>ii</sub> is still very limited.

The BiFC technique is an effective and robust tool for studying PPIs, as it enables not only the direct visualization of the occurrence and subcellular localization of PPIs in live cells (40, 41) but also the detection of weak or transient interactions due to the strong signal and high stability of the reconstituted fluorescent complex (25, 42). In the present study, we used a genome-wide BiFC-based proteomic approach to profile the TgROP18 (ROP18<sub>i</sub> and ROP18<sub>ii</sub>) interactome in human cells.







Compared with control cells, a total of 492 ROP18<sub>i</sub> and 141 ROP18<sub>ii</sub>-interacting proteins were identified. Among these proteins, six of them, NMI, IL20RB, P2RX1, IL21, UBC, and vimentin, were further confirmed as authentic ROP18<sub>i</sub> targets by our SE-FRET and Co-IP assays and, furthermore, four of them, CNBP, DCTD, NUP160, and PRAC, had been previously reported as TgROP18 substrates by a human protein array (22). All of these results confirming the TgROP18 targets have strongly supported the power of our HT-BiFC assay. In this HT-BiFC assay, the NYFP- or CYFP-tagged prey collections covered 93% of all human ORFs, facilitating a much more complete and previously unavailable description of the TgROP18 interactome. We also newly identified 488 ROP18<sub>i</sub>-interacting proteins when compared to the two previous screenings for ROP18<sub>i</sub> targets (20, 22). Such findings have demonstrated the significant advantages of the BiFC system to detect not only strong binding, but also transient or weak PPIs that would often be missed by using YTH and protein arrays (20, 22). The discovery of the novel TgROP18-interacting proteins have also shown the differences between the experimental methods used in the present study and in previous studies using YTH (20) and protein array methods (22), which analyzed the PPIs in yeast or *in vitro*. To our knowledge, ours is the first study to report the TgROP18 interactome in the natural cellular context. Our data appears to be highly complementary to the existing information about TgROP18, and the newly identified TgROP18-interacting proteins will be potential candidates for further investigations into the regulatory roles of TgROP18 in human cells.

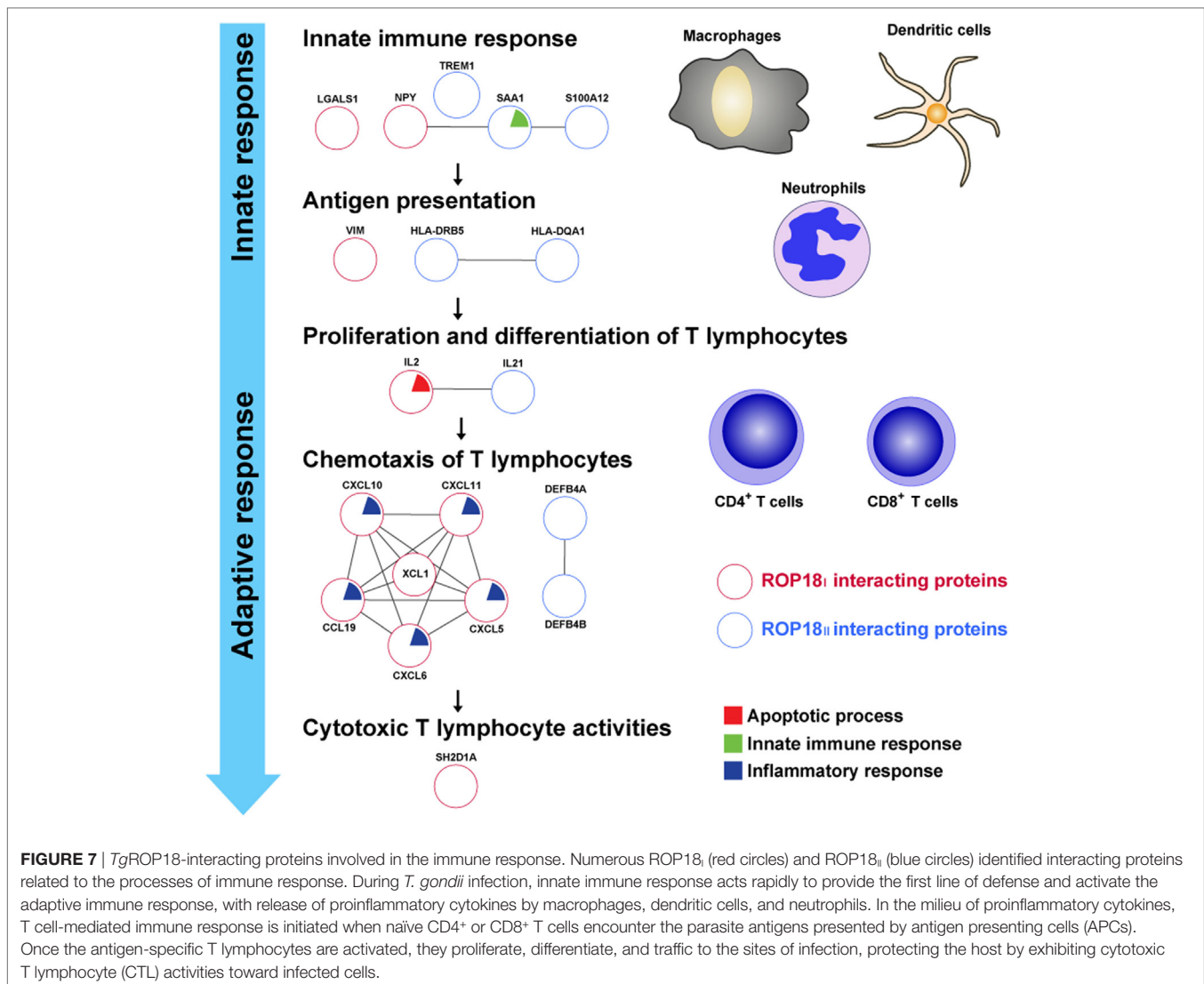
## TgROP18 and Immune Response

During *T. gondii* infection, immune defense against the parasite is strongly induced in its mammalian hosts, and T cell-mediated immune response plays a key role in this defense (43, 44). In our results, most TgROP18-interacting proteins were associated to the processes of immune response, including innate immune response, antigen presentation, activation and chemotaxis of

naïve T lymphocytes, and cytotoxic reaction of effector T lymphocytes (Figure 7).

Innate immune response provides a first line of defense against *T. gondii* infection and is essential for the activation of the adaptive immune response (45). Following the infection of *T. gondii*, innate cells, including macrophages, dendritic cells, and neutrophils, are recruited to the sites of infection, producing proinflammatory cytokines, phagocytizing the parasites, or generating reactive chemical substances in order to inhibit the replication and dissemination of the parasites (46–49). Human NPY, LGALS1, S100A12, SAA1, and TREM1 have been reported as regulators of innate immunity, modulating the innate immune functions by controlling the innate cells physiology and cytokines release (50–55). In this study, NPY, LGALS1, and TREM1 were found as targets of ROP18<sub>i</sub>, and S100A12, SAA1, and TREM1 were targets of ROP18<sub>ii</sub>. These results indicate a potential role of TgROP18 in manipulating and disarming the host innate immune response, which may contribute to the increase in parasites' survival in infected cells.

Antigen presentation is the first step for induction of T cell-mediated response (56). Infection with *T. gondii* provides a strong stimulus for antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which suggests that the parasite antigens are efficiently acquired by APCs and presented to antigen-specific T lymphocytes during infection. It has been reported that intermediate filament protein vimentin plays a key role in antigen presentation, and disruption of vimentin in Langerhans cells results in failed antigen presentation of these cells (57). HLA molecules have also been known to carry out an indispensable role in antigen processing and presentation, by binding the pathogen antigens and displaying them on the cell surface for recognition by T lymphocytes (56). In this study, we found an interaction between ROP18<sub>i</sub> and vimentin, and this interaction was confirmed by FRET and Co-IP assays (35). Moreover, HLA-DQA1 and HLA-DRB5 were identified as the targets of ROP18<sub>ii</sub>. These results suggest that TgROP18 may confer the virulence to the parasite and exert its influence on



human cellular immunity by forming PPIs with the key proteins involved in antigen processing and presentation.

Once the T lymphocytes are specifically sensitized by exposure to the parasite antigens, they undergo proliferation and differentiation, which is regulated by inflammatory cytokines (58). Proinflammatory cytokines, such as IL-2 and IL-21, are pivotal mediators in triggering development of T cell populations and effector functions against *T. gondii* infection mediated by T lymphocytes. Though the IL-2 response is not potently induced during *T. gondii* infection (59), IL-2<sup>-/-</sup> mice have a defect in production of IFN- $\gamma$  and exhibit poor CD8<sup>+</sup> T cell responses against the parasite (60). In addition, Khan et al. have reported that in mice lacking functional IL-21, expression of co-stimulatory molecules on CD8<sup>+</sup> T cells is strongly downregulated by *T. gondii* infection, and in the absence of IL-21 receptors, the functions of CD8<sup>+</sup> T cells are significantly affected (61). In the present study, we identified IL-2 and IL-21 as the host targets of ROP18<sub>i</sub>. In particular, IL-21 was further confirmed as the ROP18<sub>i</sub>-interacting protein by FRET and Co-IP assays (Figures 2 and 3). These data

suggest that ROP18<sub>i</sub> may inhibit the activation and development of host T lymphocytes by targeting the key cytokines, resulting in the dysregulation of T cell-mediated immunity.

During *T. gondii* infection, multiple chemokines are upregulated, contributing to T cells entry into the sites of infection and targeting of parasites (62). In murine ocular and cerebral toxoplasmosis, there is a significant increase in the expression levels of CXCL10 and CXCL11 over the course of infection (63, 64). CCL19 is a vital chemokine in multiple immunological processes, including generation of thymocytes, promotion of regulatory T cells activity, and homing of leukocytes (65–67). The family of  $\beta$ -defensins (DEFB) consists of a number of cationic host defense peptides, such as DEFB4A and DEFB4B, which play a dual role in both innate and adaptive immune response (68). In this study, chemokines CXCL5, CXCL6, CXCL10, CXCL11, CCL19, and XCL1 were identified as the ROP18<sub>i</sub>-interacting proteins, and DEFB4A and DEFB4B were identified as the ROP18<sub>ii</sub> targets, which indicate a regulatory role of TgROP18 in human chemokines, enabling the parasite to

interfere with the host immune responses and finally promote parasite's survival.

After being attracted to the sites of infection, the antigen-specific effector T lymphocytes display strong effector functions toward infected cells for host protection (69). SH2D1A (or signaling lymphocytic activation molecule-associated protein, SAP) is an adaptor protein that regulates signaling through signaling lymphocytic activation molecule family receptors expressed on T lymphocytes and NK cells (70). Mutations in the *sh2d1a* gene or lack of SH2D1A protein show a significant decrease in the production of IFN- $\gamma$ , resulting in disruption of cytotoxic T lymphocyte (CTL) function and defective lytic activity against EBV-positive target cells (71). In our study, we identified SH2D1A as a host target of ROP18<sub>I</sub>. Given the importance of SH2D1A in CTL activities, being targeted by ROP18<sub>I</sub> may lead to impaired function of SH2D1A, thereby decreasing cytotoxic activity against *T. gondii* infection.

## TgROP18 and Apoptosis

Apoptosis is a programmed, regulated form of cell death that permits the active and safe self-destruction of the cell (72). It plays a major role in cell development, tissue homeostasis, immune defense, and protection against tumorigenesis (73). *T. gondii* appears to use various strategies to interfere with host cell apoptosis through both pro-apoptotic and anti-apoptotic activities. Such complex dual activities of the parasite may be crucial for stable host-parasite interaction and sustained toxoplasmosis (74, 75). After acute infection, increased apoptosis of immune cells induced by *T. gondii* may suppress the immune responses against the parasite, thereby leading to immune evasion. On the other hand, inhibition of host cell apoptosis may serve as a mechanism for preserving intracellular replication and long-term survival of the parasite (76).

TgROP18 has been shown to use a variety of mechanisms, including the mitochondrial pathway, to modulate the host cell apoptosis (16). As a mitochondrial inner membrane protein, HIGD1A inhibits cytochrome c release and reduces caspases activities, thus suppressing the mitochondrial pathway of apoptosis (77). IGF1R, which is a tyrosine kinase, acts as an anti-apoptotic agent by upregulating the expression of anti-apoptotic members of the BCL2 family. Inhibition of IGF1R not just leads to reduced anti-apoptotic BCL2 proteins, but also increases expression of proapoptotic Bax/Bak-like BCL2 proteins and cleavage of caspase 3 (78). In our research, we found that HIGD1A and IGF1R were targeted by ROP18<sub>I</sub>, suggesting that these host proteins might be significant for ROP18<sub>I</sub> to manipulate human cell apoptosis through the mitochondrial pathway.

The death receptor pathway is another major pathway of apoptosis. Fas apoptotic inhibitory molecule (FAIM) is a death receptor antagonist that protects the cell from Fas-induced apoptosis by inhibiting auto-ubiquitinylation and proteasome-dependent degradation of the apoptotic suppressor protein XIAP (79). Cells overexpressing FAIM show increased resistance to apoptosis triggered by the death receptor, and suppression of FAIM expression protects the cell against death receptor-induced apoptotic cell death (80). We found an interaction between ROP18<sub>I</sub> and FAIM in this study, which suggested that

the parasite might interfere with the death receptor pathway of host cell apoptosis through targeting the key component in this pathway by TgROP18.

The ER is a central cellular organelle responsible for several crucial biological processes, and ER stress condition can trigger cell apoptosis when the stress is prolonged and severe (81). It has been reported that *T. gondii* can induce apoptosis of host cells via the ER stress pathway by upregulating the expression of C/EBP homologous protein, c-JUN NH2-terminal kinase, and activated caspase 12 (82, 83). Furthermore, ROP18<sub>I</sub> exerts a facilitated effect on the ER stress-induced apoptosis of the host cell by increasing the expression levels of the key molecules involved in the pathway (84). Consistent with this, two human proteins, PPT1 and PSMD10, which were involved in the ER stress-induced apoptosis, were identified as the ROP18<sub>II</sub>-interacting proteins in our HT-BiFC assay. PPT1 is a lysosomal enzyme that is associated with the depalmitoylation and degradation of S-acylated proteins. PSMD10, also named as p28, suppresses ER stress-induced apoptosis by upregulating the expression of GRP78 and promoting the recovery of the cell from ER stress (85). Considering the significant roles of PPT1 and PSMD10 in ER stress-induced apoptosis, disruption of PPT1 and PSMD10 by ROP18<sub>II</sub> may facilitate the ER stress-induced apoptosis of host cells, leading to restricted immune responses and high parasite burden. Our study suggests a pleiotropic role of TgROP18 in altering the host cell apoptosis through multiple targets and pathways, providing a new and better understanding of this pathological process.

## CONCLUSION

Identification of the host targets of *T. gondii* effectors is important to reveal host-parasite interaction. We used high-throughput PPI screening based on BiFC for the first time to identify the human host proteins targeted by the *T. gondii* key virulence factor TgROP18. In total, 492 and 141 human proteins were identified as the targets of ROP18<sub>I</sub> and ROP18<sub>II</sub>, respectively. These TgROP18-interacting proteins were involved in crucial pathways related to immune response and apoptosis. Our findings characterized an interactome of TgROP18 in human cells and described novel regulatory roles of TgROP18 on host cell functions. The analysis of the ROP18<sub>I</sub> and ROP18<sub>II</sub> PPIs networks would be useful to reveal the strategies of *T. gondii* virulence elicitation and the regulatory mechanisms of human responses to *T. gondii* infection.

## ETHICS STATEMENT

This article does not contain any experiments with human participants or animal subjects performed by any of the authors.

## AUTHOR CONTRIBUTIONS

JX designed and performed experiments, analyzed data, and drafted manuscript. LK performed FRET and Co-IP assays of IL20RB, and graphed data. L-JZ performed FRET and Co-IP assays of P2RX1. S-ZW performed FRET and Co-IP assays of IL21. L-JY performed FRET assay of UBC. CH performed

FRET and Co-IP assays of vimentin. CYH revised manuscript. H-JP designed experiments, revised manuscript, and submitted manuscript. All the authors read and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/articles/10.3389/fimmu.2018.00061/full#supplementary-material>.

**FIGURE S1** | Flow cytometry histograms of HTC75 cells co-expressing ROP18<sub>r</sub>- (upper panel) or ROP18<sub>r</sub>-NYFP (lower panel) and Prey-CYFP, or expressing control constructs CYFP-EV, showing the ultimate positive sorting rate was more than 90%. EV, empty vector.

**TABLE S1** | List of ROP18<sub>r</sub>-interacting proteins identified by HT-BiFC assay.

**TABLE S2** | List of ROP18<sub>r</sub>-interacting proteins identified by HT-BiFC assay.

**TABLE S3** | List of significant pathways related to ROP18<sub>r</sub> or ROP18<sub>r</sub>-interacting proteins.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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