Deactivation and mislocalization of a kinase protein induced by a single amino acid mutation on the proton transport catalytic aspartic acid

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10

Abstract

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Rhoptry protein 18 (ROP18) is a major determinant of strain-specific virulence in *Toxoplasma gondii*. The kinase activity of ROP18 is required for acute virulence, while the aspartate in the catalytic loop of ROP18 is considered essential for phosphoryl transfer. We showed that a single amino acid mutation at the catalytic aspartate residue (D409A mutation) significantly altered ROP18 kinase activity *in vitro*, and abolished ROP18-mediated ATF6 β degradation. Furthermore, the investigated single amino acid mutation in ROP18 led to alternation of subcellular localization of ROP18 protein. Structural modeling analysis suggests that these phenotypes might be associated with D409A mutation induced conformation changes in ROP18.

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Our findings demonstrate that a single amino acid mutation on the proton transport catalytic aspartic acid induced conformational alternations in ROP18 resulting in functional changes associated with ROP18 protein.

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Key words: Rhoptry protein 18, kinase activity, D409A mutation, functional changes, conformation alternation

1 Introduction

2 *Toxoplasma gondii* is an obligate intracellular parasite that causes significant
3 morbidity and mortality particularly among the newborn and the
4 immunocompromised[1]. This apicomplexan parasite engenders the common parasitic
5 disease toxoplasmosis in almost all warm-blooded animals [2]. Serologic prevalence
6 data reveal that *T. gondii* infections are highly prevalent in human population
7 throughout the world, affecting up to half of the world's population by some estimates
8 [3-5].

9
10 There are predominantly three major lineages, namely types I, II and III, among *T.*
11 *gondii* in North America and Europe[6]. Most infections in humans are caused by
12 type II *T. gondii*[7]. As in the laboratory mouse model, *T. gondii* display vastly
13 different levels of virulence among the three lineages. Type I parasites are mostly
14 lethal with an infectious dose of a single parasite, whereas type II strains have an
15 intermediate virulence that varies from 10^2 to 10^5 parasites, and type III parasites are
16 considered avirulent[8].

17
18 Genetic crosses among lineages I, II, and III were used to map the genetic loci
19 responsible for virulence. ROP5[9] , ROP18[10], and ROP16[11] were identified as
20 the major determinants of strain-specific virulence.

21
22 ROP 18 is a major determinant of virulence in type I *T. gondii*, contributing to the
23 lethal phenotype in type I lineage as well as the lack of virulence in types II and III
24 parasites [12]. ROP18 localizes to the rhoptry bulb and it is a member of the ROP2
25 superfamily [13].When parasites invade the host cells, rhoptries discharge ROP18
26 together with other ROP2 superfamily kinases to form nascent parasitophorous
27 vacuoles[14] and some into host cell cytosol [15]. Additional studies demonstrate that

1 the N-terminal arginine-rich domain of ROP 18 is critical to its function as a virulence
2 determinant[16].

3

4 Kinase activity of ROP18 is required for acute virulence by inducing host protein
5 phosphorylation and degradation. The aspartate in the catalytic loop (“HRD” motif) is
6 considered as a catalytically essential residue responsible for phosphoryl transfer[17].
7 ROPs lacking one or more of those conserved catalytic motifs, are predicted to be
8 catalytically inactive and have been termed pseudokinases[9]. ROP2/8, ROP4/7,
9 ROP5, ROP22, ROP36, ROP40, and ROP42/43/44 are identified or likely
10 pseudokinases[18,19]. Interestingly, the catalytic Asp in kinase-conserved “HRD”
11 motif is replaced with a basic residue specifically in ROP4/7 (HGK), ROP5
12 (HG[R/K/H]), ROP22 (HTH), ROP36 (HGH), ROP40 (LRR) and ROP42–43-44
13 (HGK) [9,17]. Among them, ROP5’s mechanism of action is well understood; it
14 adopts the kinase fold by binding ATP in a non-canonical conformation. Sequence
15 alignment reveals strong similarity between ROP5 and ROP18, sharing about 28% of
16 sequence identity[20].

17

18 ATF6 β was identified as a main host target of ROP18; ROP18 phosphorylates ATF6 β ,
19 thereby targeting it for proteasome-dependent degradation [21]. ATF6 β is synthesized
20 as an endoplasmic reticulum (ER)-transmembrane protein, which is activated during
21 ER stress response (ERSR) [22]. ER stress-induced proteolysis of membrane-bound
22 ATF6 liberates the N-terminal fragment of ATF6, which then translocates into the
23 nucleus and activates its target ERSR genes[23].

24

25 It is postulated herein that mutating the catalytic aspartate residue of ROP18 might
26 alter its conformation and function. Therefore, it is tested that a single amino acid
27 mutation at the catalytic aspartate residue could significantly alter ROP18 kinase
28 activity *in vitro*. Previous studies have demonstrated that this mutation could affect

intracellular parasite development and virulence[24].

ATF6 β protein level was abrogated in wild-type cells co-transfected with ROP18 but not in D409A mutants, demonstrating that kinase activity is involved in ROP18-mediated degradation. ATF6 β degradation was precluded with the addition of protease inhibitors, indicating that this particular ROP18-mediated degradation is proteasome-dependent. Colocalization of ROP18 kinase dead and ATF6 β suggested that their physical interaction occurs in the Golgi apparatus. Association of ROP18 with the cell membrane was observed, suggesting a membrane-targeting strategy for ROP18. Intriguingly, wild-type ROP18 remained membrane-bound, whereas ROP18 kinase dead was associated with intracellular trafficking vesicles and some ultimately secreted out of the cell. It is likely that changes in protein hydrophobicity or kinase activity contributed to ROP18 kinase dead's aberrant secretory pattern. It is also possible that membrane attachment is imperative for *T. gondii*'s acute virulence. Collectively, these findings indicated that a single amino acid mutation on the proton transport catalytic aspartic acid induced functional changes in ROP18, which might be associated with conformation changes within the ATP binding pocket.

Materials and Methods

Bacteria Strains, Cell lines and Plasmids

BL21 (DE3) V2RpACYC-lic-LamP-phosphatase cells (a kind gift from Dr. Raymond Hui, Structural Genomics Consortium), E. cloni® 10G cells (Lucigen, USA), XL1-Blue supercompetent cells (Agilent, USA).

Human Embryonic Kidney 293T cell (HEK 293T) was a kind gift from Dr. Paul Dear, MRC Laboratory of Molecular Biology, Cambridge, UK.

1
2 pcDNA ATF6 β -YFP; pcDNA HA_ATF6 β _YFP-T2ACFP(with a self-cleavage signal
3 [T2A] in between to produce the same level of both proteins); pcDNA HA-tagged
4 ATF6 β expression vectors were obtained from Dr. Masahiro Yamamoto, Osaka
5 University, Japan. The pGEX-6p-ROP18mat-His plasmid was a kind gift from Dr.
6 David Sibley, Washington University. The pcDNA5/FRT/TO was a kind gift from Dr.
7 Paul Dear, MRC Laboratory of Molecular Biology, Cambridge, UK. pCherry-C2
8 Rab11a and pCherry-LAMP1 mammalian expression vectors were obtained from Dr.
9 George S. W. Tsao, Department of Anatomy, University of Hong Kong.

10

11 **Chemical Bacterial Transformation**

12 50 μ l of pre-thawed competent cells were incubated with 1 μ l of plasmid on ice for
13 0.5 hours. Then the cells were heat shocked at 42 °C for 60 seconds and returned to
14 ice for 2 minutes. 250 μ l room temperature SOC medium (0.5% yeast extract, 2%
15 tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO₄, 20 mM Glucose) was added to
16 the heat-shocked transformed competent cells and incubated at 37 °C for 1 hr with
17 shaking at 220 rpm. After incubation, 20 μ l of transformation mix was spread on the
18 LB agar plate (1% Tryptone, 0.5% Yeast Extract, 1%NaCl, 1.5% agar) containing the
19 appropriate antibiotic. The plate was placed in the 37°C incubator and grown
20 overnight.

21

22 **Recombinant Protein Expression and Purification**

23 BL21 (DE3) V2RpACYC-lic-LamP-phosphatase bacteria cells were transformed by
24 chemical transformation with different plasmids. Transformed bacterial clones were
25 subsequently grown on 5ml of liquid LB (lysogeny broth) in the presence of
26 100 μ g/ml ampicillin and 30 μ g/ml chloramphenicol overnight. The bacteria culture
27 was diluted 1:100 into 500ml Terrific Broth medium (1.2% Tryptone, 2.4% Yeast

1 Extract, 0.94% K_2HPO_4 , 0.22% KH_2PO_4 , 0.5% Glycerol) containing 100 μ g/ml
2 ampicillin and 30 μ g/ml chloramphenicol and grown on a rotator shaker at 200 rpm
3 and 37°C to an OD_{600nm} of 0.6. The protein expression was induced by addition of
4 isopropyl- β -D-1-thiogalactopyranoside (IPTG; Sigma Aldrich) to a final
5 concentration of 1 mM and the cell were grown on a rotator shaker at 180rpm at 18°C
6 overnight. Cells containing the recombinant proteins were harvested by centrifugation
7 at 5000g and 4°C for 10 minutes.

8

9 The cells pellets were weighed and resuspended, lysed with 2XCellLytic B lysis buffer
10 (Sigma, USA) containing 1xComplete, Mini, EDTA-free Protease Inhibitor Cocktail
11 Tablets (Roche), Benzonase (50 units/ml; Sigma, USA), lysozyme (0.1 mg/ml; Sigma,
12 USA). The CellLytic B 2X to cell mass ratio should be 5 ml per gram of wet cell paste.
13 Then, the lysate was centrifuged at 16000 g for 10min at 4°C.

14

15 Glutathione Sepharose 4B(GE Healthcare, Sweden) was washed by PBS (140mM
16 NaCl, 2.7mM KCl, 10mM Na_2HPO_4 , 1.8mM KH_2PO_4 , pH 7.3) twice. The cell lysate
17 was incubated with the prepared Glutathione Sepharose 4B for 2h at 4 °C using gentle
18 agitation. The supernatant was carefully removed by centrifugation at 500g for 5 min
19 and the beads were then washed with PBS three times. The bound GST protein was
20 then eluted with elution buffer (50mM Tris-HCl, 10mMreduced glutathione, pH
21 8.0).Pierce BCA protein assay kit (Thermo Scientific, USA) was used to measure the
22 protein concentration, which is based on bicinchoninic acid (BCA) for the
23 colorimetric detection and quantitation of total protein.

24

25 **ADP-Glo™ Kinase Assay**

26 ADP-Glo™ Kinase Assay is a luminescent ADP detection assay to measure kinase
27 activity by quantifying the amount of ADP produced during a kinase reaction. The

1 kinase activity of ROP 18 (WT) or ROP 18(D409A) were tested according to the
2 manufacturer's instructions.

3 **Protein Separation by Sodium dodecyl sulfate** 4 **-polyacrylamide gel electrophoresis (SDS-PAGE) and** 5 **Western blot**

6 Samples for SDS-PAGE were made with sample loading buffer and sample reducing
7 agent, and heated at 95°C for 6 min before loading onto a 4-15% pre-cast
8 Mini-PROTEAN® TGX™ Precast Gel (Bio-rad, USA), which was run at 15 V/cm
9 for 60min. The running buffer was 25 mM Tris, 192 mM Glycine, 0.1 % SDS, pH
10 8.3.

11 After electrophoresis, the polyacrylamide gel was soaked in transfer buffer (25 mM
12 Tris, 192 mM Glycine, 20 % methanol (v/v)). PVDF membrane (Hybond-P;
13 Amersham Pharmacia Biotech, UK) was cut to the correct size for the gel and
14 prepared for transfer by soaking in methanol. Sponges and blotting paper were soaked
15 in transfer buffer. Place successively from the anode to the cathode: paper, the PVDF
16 membrane, the gel, and further paper taking care to remove any bubbles between the
17 different layers. The transfer cassette was loaded into the gel tank and connected to
18 power supply for 3hr at 35V. Following transfer, the membrane was incubated with 5%
19 skimmed milk prepared in TBST (TBS containing 0.1% Tween-20) for 1hr. After
20 blocking, the membrane was incubated with appropriately diluted primary antibodies
21 for 1hr at room temperature. Then the membrane was washed three times with TBST
22 for 10min. The membrane was then incubated with the secondary antibody (against
23 the primary antibody) for 1hr at RT. Then the membrane was washed three times with
24 TBST and applied with mixed detection solution A and Bin the ratio of 40:1 from
25 ECL Plus Western Blotting Detection Reagents (GE Healthcare, USA) for 1 min at
26 room temperature. Excess reagent was removed and the x-ray film was developed,
27 fixed and washed in the dark room. The protein size was estimated using Rainbow

1 Molecular Weight Marker (GE Healthcare, USA).

2

3 **Coomassie Blue Staining**

4 After electrophoresis, the polyacrylamide gel was released and fixed in fixing solution
5 (50% methanol and 10% glacial acetic acid) for 1hr with gentle agitation. The gel was
6 then stained in 0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial
7 acetic acid for 5hr with gentle agitation. The gel was destained in destaining solution
8 (40% methanol and 10% glacial acetic acid) until the background is sufficiently
9 reduced.

10

11 **ROP18 Mammalian Expression Vector Construction**

12 Restriction digests of pGEX-6p-ROP 18 plasmid DNA were completed by using
13 BamHI-HFTM and NotI-HFTM (NEB), according to the product manuals. The ROP 18
14 DNA fragment was excised and ligated with the pcDNA5/FRT/TO by using T4 DNA
15 Ligase (NEB), according to the product manuals. Then the reaction mixture was
16 transformed into E. cloni® 10G competent cells. Individual colonies were picked and
17 the plasmid was extracted for screening the incorporation of the pcDNA5/FRT/TO:
18 ROP 18 DNA construct by restriction digestion with BamHI and NotI. The
19 appropriate plasmids were sent for DNA sequencing to confirm the integrity of the
20 plasmids. The sequencing primer is : 5'-GACTTGCAGAGGGAGTCGTC -3'.

21

22 **Site-Directed Mutagenesis**

23 ROP18 D409A and ROP18 R223E mutant was generated by using QuikChange®
24 Site-Directed Mutagenesis Kit.

25 The PCR amplification was performed according to manufacturer's instructions.

26 The PCR product was treated with 10 units of Dpn I enzyme for 1 hour at 37°C to

1 digest the parental nonmutated ROP18 plasmid. 1 µl of the Dpn I-treated plasmid was
 2 transformed into XL1-Blue supercompetent cells by heat shock then the
 3 transformation reaction were plated on a LB–ampicillin agar plate and incubated
 4 overnight at 37°C.
 5 The next day, several colonies were picked and inoculated into 10 ml LB+ ampicillin
 6 medium, and incubated overnight at 37°C. The ROP18 D409A mutant plasmid was
 7 extracted by using the QIAprep Spin Miniprep Kit. The plasmids were sent for DNA
 8 sequencing to confirm the integrity of the plasmids by using the upstream primer.

10 **Cell Culture**

11 Human Embryonic Kidney 293T cell line was grown in Dulbecco's modified Eagle's
 12 medium (DMEM, (Sigma Aldrich, USA)) supplemented with 10% fetal bovine serum
 13 (FBS, (Sigma Aldrich, USA)), 100U/ml penicillin-streptomycin (Sigma Aldrich,
 14 USA), and 1% L-Glutamine (Lifetech, USA). For cell maintenance, cells were first
 15 washed with Ca²⁺, Mg²⁺-free PBS and then detached by treatment with trypsin/EDTA
 16 (0.05 % trypsin, 0.2 % EDTA) at 37°C for several minutes. As soon as the cells
 17 detached from the cell culture flask, fresh medium containing 10% FBS was used to
 18 inactivate the trypsin and the detached cells were centrifuged at 500g for 5min. The
 19 pellet was resuspended in fresh medium containing 10% FBS and the
 20 above-mentioned antibiotics. The adequate dilutions of cells were seeded in new cell
 21 culture flask.
 22 Mycoplasma contamination was monitored from time to time by using the
 23 MycoAlert™ Mycoplasma Detection Kit, which measure the luciferase signal from
 24 ATP formed by mycoplasmal enzymes.

26 **Mammalian Cells Transfection**

1 For plasmid transfection, low passage of 293T cells (5×10^5 cells/well) were seeded in
2 6-well plates one day before transfection in 10% FBS DMEM medium with
3 antibiotics. On the next day, 2 μ g of mammalian expression vectors were diluted in
4 100 μ l of pure DMEM. After mixed and 5 min incubation at room temperature, 6 μ l
5 of Fugen HD transfection reagent (Promega) was added and the mixture was
6 incubated for additional 15 min. Then the mixture was added on top of the cells drop
7 by drop. After 24-48 hrs incubation at 37°C, the transfection efficiency was checked
8 either by immunofluorescence or by Western blot analysis.

9

10 **Immunofluorescence Staining.**

11 293T cells grown on the glass cover slips were subjected to transfection treatment. At
12 24-48 h post-transfection, the cells were fixed with 4% paraformaldehyde (PFA) in
13 PBS (15 min at RT). The fixed cells were washed with PBS 3 times and
14 permeabilized with 0.3% Triton X -100 in PBS (15 min at RT). After washing with
15 PBS, the permeabilized cells were incubated with PBS.BSA for 1 h at RT. Then the
16 cells were incubated with primary antibodies against rabbit anti-ROP 18(1 : 500) and
17 mouse anti-Giantin (1:100) for 1 h. After 3 washes in PBS for 10 min each, cells were
18 then incubated to goat anti-mouse Alexa Fluor® 568 or goat anti-rabbit Dylight 350
19 conjugated immunoglobulin G (IgG) for 1 h. The cells were washed three times in
20 PBS and the coverslips were mounted on the glass slide with mounting solution.

21 For mitochondrial labeling, MitoTracker® dyes were diluted directly in the growth
22 medium at the concentration of 100nM and incubated for 30min at 37 °C. After
23 incubation, the cells were fixed in ice-cold methanol for 15 min at -20°C then rinsed 3
24 times with PBS for 10 min each.

25

26 **Fluorescence reporter assay**

27 The ATF6 β -YFP/CFP reporter plasmids were transiently co-transfected with ROP18

1 (WT) or ROP18 (D409A) into 293T cells using Fugen HD transfection reagent
2 (Roche). Fluorescence signal of transfected cells were measured using the microplate
3 reader (BMG labtech). The fluorescence intensity levels of YFP were measured at an
4 excitation wavelength of 500 ± 10 nm and an emission wavelength of 530 ± 10 nm, CFP
5 were measured at an excitation wavelength of 430 ± 10 nm and an emission
6 wavelength of 480 ± 10 nm.

8 **Immunoprecipitation**

9 293T cells grown in 6 well plates were transfected with ATF6 β together with ROP
10 18(WT) or ROP 18(D409A) plasmids for 48 h.
11 Cells were washed and resuspended in 0.5 ml of RIPA lysis buffer and protease
12 inhibitor cocktail tablets (Roche). After centrifugation, the supernatant was incubated
13 with anti-HA antibodies for 1 h. The immune complexes were recovered by
14 adsorption to Dynabeads® Protein A 1 h at room temperature. After five washes in
15 lysis buffer, the immunoprecipitates were analyzed by western blot analysis.

17 **Protein secretion analysis**

18 The cell culture medium was collected and centrifuged at 13000xg for 5 min to
19 remove the cell debris. The medium was then concentrated by 30 kDa cutoff Amicon
20 concentrators, followed the manufacturer's instructions. The concentrated solutions
21 were analysed by western blotimmunoblotting with anti- β -Tubulin and anti-ROP18
22 antibodies respectively. Non-transfection and vector only were used as the controls.
23 The cell lysates from adherent culture cells were collected as transfection controls.

25 **Results**

1 Structural modeling of wild-type ROP18, ROP18 kinase **2 dead, and homologies to known ROP18 crystal structure**

3 The crystal structure of ROP18 kinase domain has been solved [25]. By using
 4 PDBeFold(<http://www.ebi.ac.uk/msd-srv/ssm/>), a web service for identifying
 5 similarities in 3D protein structures, a comparison between predicted structures of
 6 wild-type ROP18 or ROP18 kinase dead (D409A mutant) generated from I-TASSER
 7 and the known, existing 3D structure determined by x-ray crystallography (PDB code
 8 4JRN) was made.

9 The alignment results between predicted structure and x-ray 3D structure of kinase
 10 domain are hereby listed (Table 1). The structural relatedness of the proteins involves
 11 consideration of average root-mean-square deviation (RMSD), Q score (CαG
 12 alignment) and % Sequence Identity.

13
 14 The degree of superimposability could be extrapolated from RMSD. A low RMSD
 15 between predicted structure of wild-type ROP18 and the known x-ray structure would
 16 indicate a high degree of superimposability. However, a high RMSD was obtained
 17 when comparing predicted structure of ROP18 kinase dead to predicted wild-type
 18 ROP18 structure or the known x-ray structure, suggesting a big structural divergence
 19 from both predicted wild-type ROP18 and known ROP18 crystal structure.

20
 21 Q-scores take into account the number of residues in matched secondary structure
 22 elements (SSEs) as well as positions in space. High Q-scores are obtained when
 23 comparing x-ray structure of predicted wild-type ROP18 with that of known ROP18
 24 crystal structure. This observation suggests that a large number of residues in
 25 equivalent structural elements superimpose well in three-dimensional space. However,
 26 low Q-scores are observed when comparing predicted structures of ROP18 kinase
 27 dead to that of wild-type ROP18 or known ROP18 x-ray crystallography.

1
2 There is a 61% sequence homology between the predicted kinase dead structure and
3 the x-ray 3D structure, where an 82% sequence identity between predicted wild-type
4 ROP18 models and the existing x-ray 3D structure.

5
6 The high degree of similarity between the wild-type ROP18 prediction, along with the
7 known x-ray 3D structure, indicated that I-TASSER prediction results were reliable.
8 Furthermore, D409A mutation could cause significant structural changes based on the
9 alignment model.

10
11 According to I-TASSER, amino acid residues at positions 259, 261, 262, 263, 264,
12 266, 279, 281, 356, 357, 359, 362, 413 and 416 of ROP18 protein sequence were
13 predicted ATP-binding sites. Analysis of residue-by-residue mapping data indicates a
14 high degree of similarity between predicted wild-type ROP18 and known X-ray
15 structure at the active ATP-binding site. However, misalignment of predicted D409A
16 with respect to either predicted wild-type ROP18 or existing x-ray structure at amino
17 acid residues V266 and D362 implies a significant disruption at ATP-binding sites.

18
19 Therefore, D409A mutation is hypothesized to cause conformational changes in
20 ROP18 not only on a local level but also on a global scale.

21
22 **Table 1. Multiple alignments among predicted wild-type ROP18, predicted**
23 **ROP18 kinase dead (D409A mutant), and existing ROP18 x-ray 3D structures.**
24 Structural alignments shown were conducted using I-TASSER. RMSD (Root mean
25 square deviation) of aligned structures indicates their divergence from one another.
26 The Q-score takes into account the number of residues in corresponding secondary
27 structure elements (SSEs) and their positions in space. High Q-scores are obtained for
28 structures where a large number of residues in equivalent structural elements

1 superimpose well in three-dimensional space.

2

3

4

	Structure:	Predicted D409A	Predicted wild-type	X-ray 3D structure
RMSD	Predicted D409A		2.810	2.673
	Predicted wild-type	2.810		1.758
	X-ray 3D structure	2.673	1.758	
Q-score	Predicted D409A		0.095	0.158
	Predicted wild-type	0.095		0.211
	X-ray 3D structure	0.158	0.211	
Sequence	Predicted D409A		0.662	0.611
Identify	Predicted wild-type	0.662		0.821
	X-ray 3D structure	0.611	0.821	

5

6 **Abrogation of ROP18 kinase activity by point mutation**

7 To characterize the role of the putative catalytic aspartate in the ROP18 protein kinase,

8 site-directed mutagenesis was employed to mutate the amino acid residue to alanine.

9 The necessity of the putative catalytic aspartate residue for kinase activity was

10 explored by comparing the activity of the wild-type ROP18 to that of the D409A

11 mutant. D409A mutation had similar expression level in bacteria to the wild-type

12 ROP18 (Fig 1A). Phosphorylation assays were carried out in the presence of ATP,

13 and reaction mixtures were analyzed by the ADP-Glo™ Kinase Assay Kit. In addition,

14 Western blots using anti-pThr antibody were used to examine the pThr status of the

proteins. ROP18 mutants showed a significant reduction in kinase activity, suggesting an inability to be autophosphorylated at the key Thr residues (Fig 1B).

Fig. 1. Catalytic aspartate residue is important for ROP18 kinase activity (A)

Expression level of GST-ROP18-His WT and D409A mutant protein in *E. coli*. The expressed proteins were detected by staining with Coomassie Brilliant Blue and analyzed by Western blot using anti-GST and anti-His antibodies. (B) *In vitro* kinase assays were performed using wild-type ROP18 or D409A mutants obtained by recombinant expression in *E. coli* and purification by GST pull-down. Kinase activities were measured by ADP-Glo™ Kinase Assay Kit and also analyzed by Western blot using antibody against pThr.

Alternation of degradation of ATF6β with ROP18 point mutation

It has been reported that ATF6β is a cellular host target of the *T. gondii* virulence factor ROP18. ATF6β protein is consistently reduced in cells infected with parasites harboring wild-type ROP18 but not with those lacking ROP18 (Δ rop18 strain) [21].

To confirm these findings in ROP18-transfected mammalian cells, wild-type ROP18 or D409A mammalian expression vector was constructed. YFP-ATF6β fused to CFP, with a self-cleavage signal [T2A] in between to produce the same level of both proteins, was employed in an ROP18 co-transfection experiment. Protein level of ATF6β was quantified and normalized against ratio of YFP to CFP. There was a moderate reduction of ATF6β protein levels in cells co-transfected with ROP18 kinase dead with respect to the vector control. Reduction in ATF6β protein levels was more

1 significant when cells were co-transfected with wild-type ROP18. Taken together,
2 these data suggest that kinase activity is involved in ROP18-mediated degradation
3 (Fig 2A). In parallel, lysates of 293T cells co-transfected with ATF6 β -HA and
4 wild-type ROP18 or ROP18 kinase dead were analyzed by immunoblotting.
5 ROP18-dependent decrease in ATF6 β protein level was observed when
6 overexpressing wild-type ROP18 but such phenotype was not present in D409A
7 mutant. Expression of β -Tubulin was consistent across all samples, indicating that
8 general cellular homeostasis was unaffected by ROP18 transfection (Fig 2B).

9

10 **Fig.2.Degradation of ATF6 β is associated with and dependent on ROP18 and its**

11 **kinase activity.** (A) Fluorescence readings of 293T cells co-transfected with
12 YFP-ATF6 β -CFP and ROP 18. ATF 6 β protein expression levels after co-transfecting
13 with either wild-type ROP18 or ROP18 kinase dead. Fluorescence intensity levels of
14 YFP were measured by a fluorescence microplate reader at an excitation wavelength
15 of 500/10 nm and an emission wavelength of 530/10 nm, CFP was measured at an
16 excitation wavelength of 430/10 nm and an emission wavelength of 480/10 nm.
17 Expression levels of ATF 6 β were normalized against ratio of YFP to CFP in
18 co-transfected group after 48 hours of transfection. Error bars represent means $\pm$
19 variation range of triplicates. (B) Western blot showing protein levels of
20 co-transfection with ATF6 β and either wild-type ROP18 or kinase dead. Cell lysates
21 of co-transfected 293T cells with ATF6 β -HA and either wild-type ROP18 or D409A
22 mutant were collocated at 48h post-transfection and analyzed by immunoblotting
23 against HA, ATF6 β , ROP18, and β -Tubulin. β -Tubulin was used as a loading control.

24 To investigate the mechanisms of ROP18-mediated degradation, 293T cells with
25 overexpressed wild-type ROP18 and ATF6 β -YFP were treated with MG132, which is
26 a cell-permeable proteasome inhibitor that has been reported to abrogate

ubiquitin-mediated proteasomal degradation. Untreated cells were used as a control. Compared with the empty vector control and kinase dead, degradation could be rescued in the presence of MG132, demonstrating that downregulation of ATF6 β is proteasome-dependent (Fig3A). This result was further confirmed by immunofluorescence, where there was a significant increase in ATF6 β -YFP signal intensity within ROP18-transfected cells after MG132 treatment in comparison to transfected cells with dimethyl sulfoxide (DMSO) control (Fig 3B).

These data are consistent with the previous study, confirming that kinase activity is involved in the ROP18-mediated degradation, and the degradation is in a proteasome-dependent fashion.

11

Fig. 3. ROP18-mediated degradation of ATF6 β is proteasome-dependent. (A) Proteasome inhibitor MG132 rescued ATF6 β protein degradation. ATF6 β -YFP protein was co-transfected with wild-type ROP18 or ROP18 kinase dead and treated with or without MG132. Fluorescence intensities of YFP were measured by a fluorescence microplate reader at an excitation wavelength of 500/10 nm and an emission wavelength of 530/10 nm. Error bars represent means $\pm$ the variation range of triplicates. This blot represents triplicates of two repeats. (B) Rescuing degradation of ATF6 β was substantiated by immunofluorescence. ATF6 β -YFP protein was co-transfected with wild-type ROP18 into 293T cells with or without 10 μ M of MG132 treatment. Cells were then fixed, permeabilized, and stained first with rabbit anti-ROP pAb followed by Alexa Fluor® 568 goat anti-rabbit secondary antibody then DAPI. Images were captured with a Leica fluorescence microscope at 10x objectives.

25

1 Co-immunoprecipitation assay

2 To further confirm the physical interaction between ROP18 and ATF6 β in transfected
 3 293T cells, cell lysates of co-transfected cells were analyzed by
 4 co-immunoprecipitation. Cell lysates of 293T cells transiently transfected with a
 5 plasmid encoding HA-tagged ATF6 β protein and co-transfected with either wild-type
 6 ROP18 or ROP18 kinase dead were prepared. Expressions of recombinant ATF6 β -HA
 7 and both ROP18 proteins were detected by Western blot using antibodies against
 8 ATF6 β or ROP18, respectively. Cell lysates were then immunoprecipitated with
 9 anti-HA mAb and subsequently immunoblotted with anti-ROP18 pAb to detect the
 10 recombinant ROP18. Since wild-type ROP18 mediated degradation of ATF6 β , as
 11 shown in Fig.4, ATF6 β was reasonably only detected in the sample co-transfected
 12 with ROP18 kinase dead (lane 7 of Fig 4 upper panel).

13
 14 ATF6 β is a member of the basic-leucine zipper (bZIP) DNA-binding protein family,
 15 containing an ER-transmembrane domain with the N-terminus facing the cytoplasm
 16 [22]. Upon ER stress or unfolded protein response, ATF6 β translocates from ER to the
 17 Golgi, where its cytosolic domain is released from membrane, after which it
 18 translocates once more to the nucleus to activate ER stress-specific genes [23]. The
 19 luminal domain of ATF6 β is required for sensing ER stress and permitting
 20 translocation of ATF6 β to the Golgi [26]. Collectively, these results indicated that *T.*
 21 *gondii* ROP18 protein physically binds to recombinant ATF6 β in transfected human
 22 embryonic kidney cells.

23
 24 **Fig. 4. ROP18 and ATF6 β physically interact as demonstrated by**
 25 **immunoprecipitation.** Lysates of 293T cells transiently co-transfected with 1 μ g of

1 wild-type ROP18 or ROP18 kinase dead and 1 μ g of HA-tagged ATF6 β expression
2 vectors were immunoprecipitated with antibody against HA and detected by
3 immunoblotting (IB) with antibody against ROP18 or ATF6 β . Untransfected and
4 empty vector were used as negative controls.

5

6 **Subcellular colocalization of ATF6 β and ROP18**

7 Colocalization analysis was performed in order to identify where the ROP18-ATF6 β
8 interaction occurs in transfected cells.

9 ATF6 is reported as an endoplasmic reticulum (ER) transmembrane-associated
10 transcription factor [27]. In response to ER stress, ATF6 translocates from the ER to
11 the Golgi, where it is cleaved to be activated [26, 27].

12 Previous study suggests that overexpression of ATF6 might result in levels of ER
13 chaperones being insufficient for proper folding of exogenous proteins expressed at
14 high levels. As a result, portions of endogenous ATF6 and exogenous ATF6 are
15 subjected to proteolytic processing constitutively [23].

16 To assess whether overexpressed ATF6 β is translocated into Golgi apparatus, 293T
17 cells co-transfected with pcDNA vector control or ATF6 β -YFP for 48 hours were
18 fixed for immunostaining with anti-giantin antibody, which is a known Golgi marker.
19 ATF6 β -YFP protein co-localized with giantin, thereby suggesting that ATF6 β
20 associates with the Golgi (Fig 5, upper panel).

21 Furthermore, to determine if ROP18 and ATF6 β co-localize in the Golgi apparatus,
22 cells co-transfected with ROP18, either wild-type or kinase dead, and ATF6 β were
23 analyzed in the same manner. It was established in previous experiments that
24 wild-type ROP18 could mediate ATF6 β degradation, and ATF6 β could be rescued

1 with MG132 treatment. Cells co-transfected with wild-type ROP18 and ATF6 β and
2 treated with MG132, along with cells untreated with MG132 and co-transfected with
3 ROP18 kinase dead and ATF6 β , were included in this immunofluorescence analysis.
4 In both conditions, ROP18 was mainly distributed in the Golgi apparatus, where it
5 colocalised with ATF6 β -YFP (Fig 5, lower panel).

6

7 Altogether these results suggest that *T. gondii* ROP18 protein physically interacts with
8 ATF6 β in transfected human cells and that such interaction occurs in the Golgi
9 apparatus.

10

11 **Fig.5. Subcellular colocalization of ATF6 β and ROP18 in Golgi compartment**
12 **within transfected cells.** 293T cells co-transfected with wild-type ROP18 or ROP18
13 kinase dead and ATF6 β , with or without MG132 treatment, were analyzed by
14 immunofluorescence assay. Giantin (Red), ATF6 β -YFP (Green), ROP18 (Blue) were
15 stained using antibodies specific to giantin (AlexaFluor 568) and ROP18 (Dylight 350)
16 respectively. Images were captured with a Zeiss fluorescence microscope using 63x
17 objectives.

18

19 **Alternation of subcellular localization of ROP18 protein**
20 **with single amino acid mutation**

21 Intriguingly, while most ROP18 protein was present in the Golgi, some was evident
22 on the plasma membrane (Fig 5, lower panel). To further investigate subcellular
23 localization of ROP18 protein, immunofluorescence staining was performed on cells
24 transfected with wild-type ROP18 and ROP18 (D409A), with or without

1 permeabilization, 24 and 48 hours post-transfection. At 24h post-transfection, both
2 wild-type ROP18 and ROP18 (D409A) were distributed both along cell membrane
3 and in an intracellular compartment close to the perinuclear region – the Golgi
4 compartment. Nevertheless, at 48 h post transfection of ROP18 kinase dead, ROP18
5 was not only found inside Golgi compartments and on cell surface but also observable
6 in small cytosolic patches, which could be intracellular trafficking vesicles (Fig 6).
7 An inability of those cytosolic patches to colocalize with mitochondrial,
8 lysosomal-associated membrane protein 1 (LAMP1) and Rab11 (a recycling
9 endosomes marker) suggests that said cytosolic patches might be secretory vesicles
10 (S1 Fig).

11

12 **Fig.6.Subcellular localization of ROP18 in transfected 293T cells.** Cells were
13 transfected with wild-type ROP18 or ROP18 kinase dead plasmid for 24hr and 48 hr.
14 Cells were fixed, permeabilized or not with Triton X-100 and stained with rabbit
15 anti-ROP pAb and followed by Alexa Fluor® 568 goat anti-rabbit secondary antibody.
16 Images were captured with a Zeiss fluorescence microscope using 63x objectives.

17

18 To ascertain whether those cytosolic patches are secretion vesicles, wild-type
19 ROP18-and ROP18 kinase dead-transfected cultures' supernatants were collected,
20 concentrated by Amicon concentrators, then analyzed by SDS-PAGE followed by
21 Western blot with antibody against β -Tubulin or ROP18. Cell pellets were collected as
22 control to monitor transfection. Interestingly, ROP18 was present in supernatant of
23 ROP18 kinase dead-transfected culture but not in supernatant of wild-type
24 ROP18-transfected cells. This indicated that ROP18 D409A was secreted. Failure to
25 detect β -Tubulin protein in all supernatants suggested no contamination of
26 intracellular protein (Fig 7).

Fig. 7. ROP18 kinase dead is released into culture supernatant. Supernatant from cell culture is abbreviated as S/N; C/P is cell pellets. Detection of ROP18 protein in cell culture medium was performed with antibody against ROP18. HEK 293T cells were transfected with empty vector, ROP18 kinase dead or wild-type ROP18 plasmid. Cell culture medium and cell pellets were collected. Culture medium was centrifuged at 13000xg for 5 min to remove the cell debris and concentrated by a 30 kDa-cutoff Amicon concentrator and analyzed by Western blot.

Discussion

Structural homologies among predicted structures of wild-type ROP18 and ROP18 kinase dead, as well as the known x-ray structure of ROP18, were analyzed by PDBeFold, which is an interactive service for comparing three-dimensional protein structures. Misalignment of protein structures suggests little structural similarity between wild-type ROP18 and ROP18 kinase dead. Furthermore, this point mutation might cause conformational changes not only on a local level, particularly at the proton transport catalytic aspartic acid, but also on a global level in the ROP18 protein.

Site-directed mutagenesis was employed to mutate the critical active site aspartate to alanine to create the D409A mutation. As expected, the single amino acid mutation did not significantly change the protein expression level. On the other hand, the D409A mutant displayed a significant reduced kinase activity *in vitro* and it showed an inability to be autophosphorylated at the key threonine residues.

To better characterize wild-type ROP18 and ROP18 kinase dead, ATF6 β protein level was consistently reduced in ROP18 co-transfected cells. In addition, reduction in

1 ATF6 β protein levels was more significant in cells co-transfected with wild-type
2 ROP18 compared to those co-transfected with ROP18 kinase dead, suggesting that
3 kinase activity is involved in ROP18-mediated degradation. Proteasome inhibitor
4 MG132 was able to rescue ROP18-associated degradation, suggesting that
5 downregulation of ATF6 β process is proteasome-dependent.

6 These data confirm that kinase activity is indispensable for ROP18-mediated
7 degradation, and the point mutation at key catalytic residue could abolish ROP18's
8 function.

9 Cells have developed signaling pathways to monitor folding environment of the ER
10 and adjust the ER folding capacity accordingly. When unfolded or misfolded proteins
11 accumulate in the ER, signals are transmitted from the ER to the nucleus and
12 cytoplasm. These pathways are termed the ER stress or unfolded protein response.
13 ATF6 β , a primary ER stress sensor, initiates several cellular responses and signaling
14 pathways to restore ER homeostasis.

15

16 *T. gondii* parasitophorous vacuole membrane (PVM) was reported to form tight
17 associations with host mitochondria and the ER. Therefore, it is plausible that
18 ER-localized ATF6 β in vicinity of PVMs might gain access to ROP18. Indeed,
19 ATF6 β -YFP and CFP-tagged ROP18 kinase dead were colocalized with ER-specific
20 RFP in transfected 293T cells, suggesting that ROP18 is in close proximity to proteins
21 localized in the ER. HFFs infected with parasites were analyzed by electron
22 microscopy, pointing out that PVMs directly fuse with the host ER [21].

23

24 Earlier research suggests that *T. gondii* infection could trigger unfolded protein
25 response [28]. Previous study also demonstrates that overexpressing HA-ATF6 β
26 results in activation of unfolded protein response in 293T cells; however,
27 co-expression of ROP18 downregulates ATF6 β -dependent activation in a

1 dose-dependent manner [21].

2 In response of ER stress, ATF6 translocates from the ER to the Golgi, where it is
3 cleaved and processed to its active form [26, 27]. ATF6 has been shown to be
4 processed by Site-1 protease (S1P) and Site-2 protease (S2P), both of which require
5 RxxL and asparagine/proline motifs [29]. Coincidentally, ROP18 sequence has
6 RMGL and NREP motifs, both of which might be recognized and processed by S1P
7 and S2P at Golgi. ROP18 does undergo cleavage, and its post-cleavage form was
8 detected by the polyclonal antibody against ROP18, as shown in Fig 2B. The
9 post-cleavage ROP18 interacted with ATF6 β protein in the co-transfected cells. Taken
10 together, these data suggest that ROP18 might migrate to the Golgi and also interact
11 with ATF6 β .

12 Colocalization of ROP18 kinase dead and ATF6 β confirmed that the interaction might
13 also occur in the Golgi apparatus. Overexpressed ROP18 could translocate into Golgi,
14 where ATF6 β is known to be cleaved and processed, suggesting that this transient
15 interaction starts from ER to the Golgi. Since the Golgi serves as a major
16 protein-sorting hub for secretory cargos, it receives *de novo* synthesized proteins from
17 the ER destined for secretion from the cell or for residence in endocytic branches of
18 the secretory pathway or along the plasma membrane [30]. In order to determine
19 whether overexpressed ROP18 could migrate to cell membrane from the Golgi,
20 immunofluorescence staining on ROP18 transfected cells, with or without
21 permeabilization, was performed. Association of ROP18 with cell membrane was
22 observed, suggesting a membrane-targeting strategy for ROP18.

Being so instrumental to *T. gondii* pathogenesis, then ascent parasitophorous vacuole membrane is believed to derive from host cell plasma membrane [31]. This fact is consistent with the finding that ROP18 localizes to early- and late-stage parasitophorous vacuole membranes [32]. In addition to cell membrane localization, some ROP18 kinase dead was found located inside intracellular trafficking vesicles, and further experiment demonstrated that some ROP18 kinase dead was secreted into culture medium.

Whether there is any correlation between eliminating kinase activity and localization or secretory pattern remains to be elucidated. However, it is plausible that altering kinase activity itself or changing protein hydrophobicity and hydrophilicity as a result of mutations engenders protein mislocalization.

Collectively, findings presented herein demonstrate that a single amino acid mutation on the proton transport catalytic aspartic acid induced functional changes associated with ROP18 protein, and these changes are very likely associated with conformational alterations in the secretory protein.

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A:

B:

Fig. 1. Catalytic aspartate residue is important for ROP18 kinase activity

Fig.2. Degradation of ATF6 β is associated with and dependent on ROP18 and its kinase activity.

A:

B:

Fig.3.ROP18-mediated degradation of ATF6 β is proteasome-dependent.

Fig.4.ROP18 and ATF6 β physically interact as demonstrated by immunoprecipitation.

Fig.5.Subcellular colocalization of ATF6 β and ROP18 in Golgi compartment within transfected cells.

Fig.6. Subcellular localization of ROP18 in transfected 293T cells.

Fig. 7. ROP18 kinase dead is released into culture supernatant.