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1 **The *Toxoplasma gondii* inhibitor-2 regulates protein phosphatase 1**
2 **activity through multiple motifs.**

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1 **ABSTRACT**

2 *Toxoplasma gondii* has a complex life cycle characterized by multiple differentiation steps that
3 are essential for its survival in both human and definitive feline host. Several studies have
4 demonstrated the importance of phosphorylations by protein kinases during the life cycle of *T.*
5 *gondii*. However, very little is known about protein phosphatases and their regulators in the
6 parasite. We report the molecular and functional characterization of the *T. gondii* ortholog of
7 the inhibitor-2 protein, designated TgI2. We show that TgI2 encompasses conserved motifs
8 involved in the interaction and modulation of the phosphatase activity of *T. gondii* protein
9 phosphatase 1, named TgPP1. We show that a specific combination of motifs is involved in
10 binding and/or inhibition of the TgPP1 activity. We show here that the TgI2 protein is a potent
11 inhibitor of TgPP1 phosphatase activity. TgI2 SILK and RVxF motifs are critical for regulating
12 the activity of TgPP1, a feature that is common with the higher eukaryotes inhibitor-2 protein

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14 **Key words: Toxoplasma; phosphatase; PP1; inhibitor-2.**

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1 INTRODUCTION

2 *Toxoplasma gondii* is a unicellular eukaryote that infects around a third of the human population
3 worldwide. This parasite belongs to the Apicomplexa phylum which contains other deadly
4 parasite such as *Plasmodium* (the causative agent of malaria) and *Cryptosporidium* (responsible
5 for cryptosporidiosis). *T. gondii* is of clinical importance especially in pregnant women. The
6 first infection can potentially cause severe illness and death in the developing fetus. Moreover,
7 the opportunistic nature of this parasite can lead to encephalitis in immunocompromised
8 individuals and ocular diseases in immunocompetent or immunosuppressed patients (Weiss and
9 Dubey, 2009). *T. gondii* is acquired primarily through the ingestion of sporulated oocysts
10 containing sporozoites, which are shed by the definitive host (felids) or by ingestion of cysts-
11 contaminated meats. In the gastrointestinal tract, the sporozoites or bradyzoites present in the
12 cysts are released and can invade the intestinal epithelium where they differentiate in rapidly
13 dividing tachyzoites. Those tachyzoites disseminate and proliferate during the acute stage of
14 the infection before differentiating in bradyzoites, which encyst in several organs such as
15 muscle tissue or in the central nervous system establishing a chronic infection (Blader *et al.*,
16 2015). Thus *T. gondii* has a complex life cycle which is characterized by multiple differentiation
17 steps that are essential for its survival in both human and definitive feline host.

18 Several studies have demonstrated the importance of phosphorylations by protein kinases
19 during the life cycle of *T. gondii*, from invasion (Sugi *et al.*, 2010) to egress (Moudy *et al.*,
20 2001). In *T. gondii* tachyzoites, a few protein kinases have been shown to play a role in cell
21 division during endodyogeny (Suvorova *et al.*, 2015)(Berry *et al.*, 2016)(Morlon-Guyot *et al.*,
22 2014). However, very little is known about protein phosphatases (PPs) and their regulators in
23 the parasite. In other eukaryotes, the protein phosphatase type 1 (PP1), is considered as one of
24 the major phosphatase involved in the control of many dephosphorylation steps. This
25 serine/threonine phosphatase, which exhibits a high degree of conservation throughout

1 evolution, is known to be involved in numerous essential cellular processes including protein
2 synthesis, actin organization, transcription and cell cycle progression (Ceulemans and Bollen,
3 2004). It is now known that PP1 localization, activity, and/or specificity are controlled by a
4 large number of interacting-proteins (Peti and Page, 2015). Functional and structural studies
5 revealed that interactions between PP1 and its regulators are mediated by specific binding sites
6 that are conserved throughout evolution, including the so called RVxF and SILK motifs
7 (Wakula *et al.*, 2003)(Huang *et al.*, 1999). So far, about 200 regulators have been described in
8 humans (Hendrickx *et al.*, 2009) while 100 proteins potentially interact with PP1 in
9 *Plasmodium* (Hollin *et al.*, 2016).

10 In *T. gondii*, phosphatase inhibitors were used as a proxy to measure the biological role of
11 phosphatases activity. In particular, the use of okadaic acid, an inhibitor of PP2A and PP1, lead
12 to a decreased invasion of the treated tachyzoites suggesting an important role of PP1 in *T.*
13 *gondii* biology (Delorme *et al.*, 2002). Moreover and similarly to *Plasmodium falciparum*, only
14 one gene encoding TgPP1 has been identified (Daher *et al.*, 2007). In this study, the authors
15 identified a protein (TgLRR1) homolog to the yeast sds22 protein, known to regulate the yeast
16 PP1 activity (Hong *et al.*, 2000). TgLRR1 belongs to the leucine-rich repeat protein family and
17 interacts with TgPP1 *in vitro* leading to the inhibition of its activity (Daher *et al.*, 2007).

18 Other interactors are also conserved and well described in the literature. For example, a small
19 protein called inhibitor-2 (I2) was shown to play an important role in cell cycle regulation of
20 yeast and human (Tung *et al.*, 1995)(Wang *et al.*, 2008). Likewise, an homolog of this protein
21 was identified in *P. falciparum* (Fréville *et al.*, 2013). This study suggested an essential role for
22 Pfl2 for normal growth of the parasite (Fréville *et al.*, 2013).

1 In the present study, we report the molecular and functional characterization of the *T. gondii*
2 ortholog of Pfl2, designated TgI2. We show that TgI2 encompasses conserved motifs involved
3 in the interaction and modulation of TgPP1 activity.

4

5 **MATERIALS AND METHODS**

6 *Molecular cloning of TgPP1 and TgI2*

7 The full length coding region of TgPP1 and TgI2 were amplified by PCR using the primers P1-
8 P2 and P3-P4 (Table S1). PCR products were then subcloned in a pJET1.2/blunt vector
9 (ThermoFisher) and verified by sequencing for the absence of any modification introduced by
10 Taq polymerase. For the expression of TgPP1 and TgI2, pET28a (Novagen) and pGEX 6p3
11 (GE Healthcare) expression system were used respectively. Restriction sites are mentioned in
12 Table S1.

13 To obtain the TgI2 mutant constructs, we performed a PCR-based site-directed mutagenesis
14 strategy using the pGEX 6p3-TgI2 as template, the primers P5-P6 (TgI2K6A) or P7-P8
15 (TgI2W37A) or P9-P10 (TgI2R121A/K122A) and Phusion polymerase (NEB). The PCR
16 conditions consisted of 30s at 98°C followed by 16 cycles at 98°C (5s), 55°C (15s) and 72°C
17 (7min). The parental DNA plasmid was then digested with DpnI. Mutated plasmids were
18 verified by sequencing for the replacement of lysine 6, tryptophan 37, arginine 121 and lysine
19 122 by an alanine and then used for the expression of mutant TgI2 recombinant proteins.

20 *Recombinant proteins expression*

21 Protein expression was carried out in the *Escherichia coli* BL21 strain. For the expression of
22 TgPP1, culture was induced overnight at 16°C in the presence of 0.5mM isopropyl β -D-1
23 thiogalactopyranoside (IPTG) and 1mM MnCl₂. Cells were harvested in sonication buffer

1 (20mM Tris, 150mM NaCl, 1% Triton X-100, lysozyme 1mg/ml, 1mM phenylmethylsulfonyl
2 fluoride (PMSF). 6His-tagged TgPP1 proteins were purified according to manufacturer's
3 instructions by Ni²⁺ chelation chromatography (Macherey-Nagel). The extract was prepared
4 using a buffer containing 20mM Tris HCl (pH 7.5), 150mM NaCl, 20mM Imidazole and 1mM
5 MnCl₂ and loaded on a 1 ml nickel-NTA (Nitrilotriacetic acid) resin column (Macherey Nagel).
6 Elution steps were performed with a buffer containing 20mM Tris HCl (pH 7.5), 500mM NaCl,
7 400mM Imidazole and 1mM MnCl₂. The imidazole-eluted proteins were dialyzed against the
8 following buffer: 20mM Tris HCl (pH 7.5), 500mM NaCl and 1mM MnCl₂. For the expression
9 of the different forms of TgI2 (wild-type and mutated), cultures were induced at 37°C for 3h in
10 the presence of 0.5mM IPTG. GST-tagged proteins were then purified according to
11 manufacturer's instruction using glutathione-agarose beads (Macherey Nagel). Elution steps
12 were performed with a 10mM glutathione, 50mM Tris HCl (pH 8) buffer. Under these
13 conditions, the purity checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis
14 (SDS-PAGE) followed by Instant Blue™ (Expedeon) was >80%.

15 Prior to phosphatase activity assays, GST-tagged proteins have been incubated in a buffer
16 containing 50mM Tris HCl (pH 7), 150mM NaCl, 1mM EDTA and 1mM dithiothreitol (DTT)
17 following the washing steps. After addition of 7 units of PreScission protease (GE Healthcare),
18 the mixture is incubated for 6h at 4°C allowing the cleavage of the glutathione S-transferase
19 (GST).

20 *GST pull down assays*

21 Purified TgI2-GST recombinant protein (wild-type or mutated) were bound to glutathione-
22 agarose beads 2h at 4°C on a rotating wheel and washed with a binding buffer containing 20mM
23 HEPES (, 20mM Tris HCl (pH 7.5), 500mM NaCl, 0.2mM EDTA, 1mM MnCl₂, 0.1% Triton
24 X-100, 1mM PMSF and 25µg of bovine serum albumin (BSA). Ten µg of TgPP1-6His

1 recombinant protein were incubated with the TgI2-GST or GST bound to glutathione-agarose
2 beads in binding buffer for 2h à 4°C on a rotating wheel. After 5 washes with binding buffer,
3 proteins were eluted in SDS-PAGE buffer and loaded on a denaturing 15% polyacrylamide gel.
4 Separated proteins were then blotted to nitrocellulose. Blots were revealed with anti-His and
5 anti-GST antibodies. Horseradish peroxidase labeled anti-mouse or anti-rabbit
6 immunoglobulins (1:5000) were used as secondary antibodies followed by chemiluminescence
7 detection (Pierce).

8 *TgPP1 activity assay*

9 The activity of recombinant TgPP1 with p-nitrophenyl phosphate (pNPP) as substrate was
10 assayed as previously described (Daher *et al.*, 2007). Initial experiments were performed to
11 determine the optimal conditions for TgPP1 activity. The activity was assessed in a reaction
12 containing 50mM Tris-HCl (pH 7.5), 50mM HEPES, 100mM NaCl, 1mM DTT, 1mM MnCl₂
13 and 0.1mM Brij35. The reaction was started by adding 5mM of pNPP. After incubation 1h at
14 37°C, phosphatase activity was determined spectrophotometrically at 405nm.

15 To investigate the role of TgI2 recombinant proteins on TgPP1-6His activity, various
16 concentrations of proteins were added to TgPP1 recombinant protein and preincubated 30min
17 at 37°C before testing the TgPP1 activity. GST was used as control.

18 *Parasite tissue culture and manipulation*

19 *T. gondii* strain RHΔKu80 tachyzoites were propagated *in vitro* in human foreskin fibroblasts
20 (HFF) using Dulbeccos's modified Eagles medium supplemented with 10% fetal calf serum
21 (FCS), 2mM glutamine, and 1% penicillin-streptomycin (100Units/μL of penicillin and
22 100μg/μL of streptomycin). *T. gondii* tachyzoites were grown in ventilated tissue culture flasks
23 at 37°C and 5% CO₂. Transgenes were introduced by electroporation into tachyzoites of *T.*

1 *gondii* strains and stable transformants were selected by culture in the presence of 2 μ M
2 pyrimethamine. Clonal lines were obtained by limiting dilution.

3 *Antibodies*

4 The anti-TgENO2 rabbit, anti-TgAlba1 mouse and anti-TgMIC1 mouse antibodies were used
5 at 1:1000 dilution. The anti-HA rabbit antibody (Cell signaling) was used at 1:500 in
6 ImmunoFluorescence assay (IFA) and in Western blots.

7 *Immunofluorescence Assay and confocal imaging*

8 Intracellular tachyzoites were fixed with 4% paraformaldehyde in PBS for 15min, followed by
9 two PBS washes. Parasites were permeabilized with 0.1% Triton X-100 in PBS containing
10 0.1% glycine for 10min at room temperature. Samples were blocked with 3% FCS in the same
11 buffer and the primary antibodies were added on parasites in the same buffer for 1h at room
12 temperature. Secondary antibody coupled to Alexa-488 or to Alexa-594 diluted at 1:1000 were
13 added together with DAPI (4',6-diamino-2-phenylindole) for nucleus staining. Confocal
14 imaging was performed with an LSM880 microscope (Zeiss) and a Plan Apochromat objective
15 (Plan-Apochromat 63x:1.40 Oil DIC M27, Zeiss).

16 *Statistical analysis*

17 The Student t-test was performed to assess the statistical significance of the results.

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1 RESULTS

2 *Toxoplasma gondii* I2 protein encompasses conserved motifs.

3 In order to expand our knowledge of TgPP1 regulators repertoire in *T. gondii*, we searched the
4 ToxoDB database (Gajria *et al.*, 2008) for a homolog of the Inhibitor-2 (I2) protein, a well know
5 regulator of PP1 in numerous organisms. We performed a Basic Local Alignment Search Tool
6 (BLAST) analysis (Altschul *et al.*, 1990) using the Pfl2 protein sequence and found a protein
7 whose sequence showed 31 % identity on the full length sequence. We aligned the TgI2
8 sequence with those of the human and *P. falciparum* homologs (Fig. 1A) using the MultAlign
9 software (Corpet, 1988). Using the alignment shown in Fig. 1A and the location of known PP1
10 interacting motifs present on the human and Pfl2 proteins we identified three putative PP1
11 binding motifs in the TgI2 sequence (Fig. 1, boxed). In Pfl2, only two motifs were identified
12 and shown to have an impact on its binding to PfPP1 (Fréville *et al.*, 2013), while three
13 functional motifs are found in the human I2 protein (Bollen *et al.*, 2010). By sequence
14 homology and according to the alignment shown in Fig.1A, we found in the TgI2 sequence a
15 first motif, ³VIGK⁶, potentially corresponding to a variation of the sequence of the SILK motif
16 consensus ([SG]ILK). The second motif, ³³KHLTW³⁷, matched the consensus sequence for
17 the RVxF motif ([RK]x[VI]x[FW]) with the exception of the valine or isoleucine position. In
18 the human I2, this particular residue is also changed (Fig. 1A) with no impact on the binding
19 properties of this RVxF motif variant (Huang *et al.*, 1999). Finally, we found a third motif
20 (¹¹⁷FELKRKQHY¹²⁵) that matches the consensus sequence for the FKK/HYNE motif
21 (FxxR/KxR/KxHY).

22 To better understand the distribution of these PP1 binding motifs among eukaryotic homologs
23 of TgI2, we gathered the sequence of these proteins in apicomplexan parasites, kinetoplastids,
24 plants and few Opisthokonts and identified their putative PP1 binding motifs (Fig. 1B).

1 Surprisingly, TgI2 is the only apicomplexan I2 protein to encompass a SILK-like motif, which
2 is found in most Opisthokonts (Human, mouse or Drosophila) but not in plants (Arabidopsis)
3 or Kinetoplastids (Trypanosoma and Leishmania) homologs of the I2 protein. (Fig. 1B).

4

5 ***Toxoplasma gondii* I2 protein interacts with TgPP1 through the RVxF motif.**

6 To test the ability of the TgI2 protein to bind TgPP1, we produced both recombinant proteins
7 with a GST and 6His-tag respectively (Fig. S1A and B). We also produced mutated forms of
8 the TgI2 protein for which each putative motif was mutated (Fig. S1C). To investigate the
9 respective role of each motif, we produced a combination of each mutation, yielding to a total
10 of 7 mutants for the TgI2 protein. We also checked the purity of each TgI2 mutants and
11 proceeded to a GST-pull down assay (Fig. S1D). In a binding assay, we incubated TgPP1-6His
12 with wild-type or each TgI2-GST mutant coupled to Glutathione-Agarose beads and washed
13 them extensively. We then performed a Western-blot on the eluates of each binding-assay using
14 an anti-6His antibody to identify the TgPP1 protein. We used a Western-blot with an anti-GST
15 antibody to verify the quantity of each TgI2 protein that was originally on the beads. Using this
16 protocol, we showed that the wild-type TgI2 protein is able to bind to TgPP1 (Fig. 2A – lane 3)
17 while the GST alone did not yield detectable TgPP1 signal (Fig. 2A – lane 2). We also showed
18 that the mutation of SILK-like or HYNE motifs of TgI2 protein (Fig. 2A – lanes 4 and 6,
19 respectively) had little change in its TgPP1 binding capacity. In contrast, the mutation of the
20 RVxF motif exhibited a drastic effect, with the TgPP1 signal level close to the background (Fig.
21 2A – lane 5). The combinatorial mutation of both HYNE and SILK-like resulted in similar
22 results than those obtained with the WT TgI2 (Fig. 2A – lane 8) suggesting that the RVxF motif
23 is the major motif for TgPP1 binding.

1 While the interaction with TgI2 encompassing any mutation combined with the mutated RVxF
2 resulted in small but close to the background levels of TgPP1 (Fig. 2A – lane 7 and 9), the
3 interaction with the TgI2 protein mutated for the three motifs produced no detectable TgPP1
4 (Fig. 2A – lane 10). These results suggest that both SILK-like and HYNE motif may contribute
5 to the TgPP1 binding and are therefore secondary binding motifs.

6 We repeated this experiment three times and measured the intensity of the TgPP1 band for each
7 interaction. We also measured the quantity of TgI2 for each lane in order to normalize for the
8 quantity of TgI2 protein that was initially on the beads. We therefore calculated a ratio of the
9 intensity of the TgPP1 band over the intensity of the GST-tagged protein that reflects the ability
10 of each GST-tagged protein to bind to TgPP1 (Fig. 2B). These ratio calculated for the mutant
11 lacking the SILK-like and/or HYNE motif indicate that they bind to TgPP1 in a similar manner
12 as the WT TgI2. In contrast, the mutants lacking the RVxF motif show a ten-fold reduction of
13 their TgPP1-binding capacity while the GST and the triple mutant lack the ability to bind to
14 TgPP1 (Fig. 2B). Therefore, these results indicate that the RVxF motif is a major contributor
15 to the TgPP1 binding ability while the HYNE and SILK-like motifs only show a residual
16 contribution.

17 **TgPP1 is an active phosphatase inhibited by okadaic acid.**

18 To verify if the TgPP1-6His protein we produced was active in our experimental conditions,
19 we performed a colorimetric phosphatase assay using a small phosphorylated peptide. Using
20 the same quantity as starting material, we compared the TgPP1 activity to that of a commercial
21 rabbit PP1 (Fig. 3A). These data showed a robust phosphatase activity increased with
22 concentration of the PP1 protein. TgPP1 activity is undistinguishable from that of the rabbit
23 PP1. When TgPP1 incubation buffer lacked $MnCl_2$, a cation that is indispensable for the PP1
24 phosphatase activity, we found that the activity measured was independent from the

1 concentration of TgPP1 added, indicating that TgPP1 activity is dependent on the presence of
2 MnCl₂.

3 To ensure that the phosphatase activity measured in the previous assay was due to a PP1-like
4 phosphatase, we incubated TgPP1 and PfPP1, as a control, in increasing concentration of
5 okadaic acid and measured their phosphatase activity (Fig. 3B) [14]. We found that both TgPP1
6 and PfPP1 phosphatase activity were inhibited by okadaic acid. We measured an 80 %
7 inhibition of their activity without inhibitor at the highest concentration of okadaic acid.
8 Therefore, the TgPP1 produced in our experimental condition shows a measurable phosphatase
9 activity.

10 **TgI2 acts as an inhibitor of TgPP1 phosphatase activity through its RVxF and SILK**
11 **motifs.**

12 To test the regulatory function of TgI2 on TgPP1, we performed a phosphatase activity assay
13 in presence of increasing concentration of wild-type TgI2. While the same concentrations of
14 GST alone were not able to induce any significant change in TgPP1 (Fig. S2), increasing
15 concentration of wild-type TgI2 led to a 50 % inhibition of TgPP1 activity (Fig. 4A).

16 To test the effect of each PP1 binding motif on the inhibitory activity of TgI2, we incubated
17 TgPP1 with increasing concentration of mutated TgI2. First, we tested TgI2 mutated for the 3
18 binding motifs and found that this fully mutated version of TgI2 is unable to significantly alter
19 the TgPP1 activity (Fig. 4B).

20 In good concordance with the binding-assay, the TgI2 protein mutated for the SILK-like (Fig.
21 4C) or the HYNE (Fig. 4D) motif alone, kept their ability to inhibit TgPP1 activity.
22 Surprisingly, the single mutation of the RVxF motif was not sufficient to abolish the inhibitory
23 effect of TgI2 on TgPP1 activity (Fig. 4E) although the inhibition level was decreased compare
24 to the wild-type TgI2, suggesting that the SILK-like and/or the HYNE motif are able to convey

1 the inhibitory function of TgI2. To identify whether both SILK-like and HYNE motifs are
2 necessary for the TgI2 inhibition of TgPP1 activity, we used the TgI2 mutants where two out
3 of three motifs are mutated. The TgI2 protein encompassing only the unmutated HYNE motif
4 was unable to inhibit TgPP1 activity, suggesting that this motif alone is not sufficient for the
5 inhibitory function of TgI2 (Fig. 4F). These results indicate that the SILK-like motif alone may
6 be able to mediate the inhibition of the TgPP1 activity. This was confirmed when using a TgI2
7 mutant for both HYNE and RVxF motifs, which contained the SILK-like motif alone (Fig. 4G).
8 Indeed, this mutant TgI2 protein was able to inhibit the TgPP1 activity (Fig. 4G), indicating its
9 functional role in the TgPP1 activity inhibition.

10 Finally, we verified whether the TgI2 protein mutated only in the RVxF motif was able to
11 inhibit the TgPP1 activity. In this assay, the TgI2 protein mutated for both HYNE and SILK-
12 like motif was able to significantly inhibit the TgPP1 activity (Fig. 4H), as suggested by the
13 binding-assay.

14 **TgI2 is located both in the nucleus and the cytoplasm of tachyzoites.**

15 To gain more information about the function of TgI2 in the biology of the parasite, we produced
16 an epitope-tagged TgI2-HA strain. We used this strain in IFA and colocalized TgI2-HA with *T.*
17 *gondii* cellular markers. First, we used a nuclear marker (TgENO2) (Ferguson *et al.*, 2002) and
18 performed an IFA on the TgI2-HA strain. The Fig. 5A shows the colocalization of these proteins
19 and demonstrate that the signal from the HA-tag is localized in the nucleus as well as the
20 cytoplasm. This was further confirmed by the quantitative fluorescence intensity plot that shows
21 that the HA signal is distributed in the nucleus and cytoplasm compartments (Fig. 5B). To
22 confirm this hypothesis, we used a cytoplasmic marker (TgAlba1) (Gissot *et al.*, 2013) in IFA
23 together with an anti-HA antibody. In Fig. 5C, we show a strong codistribution of the signal
24 given by the HA-tag and the cytoplasmic marker. This was further confirmed by the quantitative

1 fluorescence intensity plot showing that most of the HA signal is localized in the cytoplasm but
2 also in the nucleoplasm (Fig. 5D). To further describe TgI2 localization, we performed a co-
3 staining using a microneme marker (TgMIC1). Micronemes are secretory organelles that reside
4 at the apical end of the parasite. We showed that TgI2-HA does not codistribute with
5 micronemes (Fig. S3).

6 **DISCUSSION**

7 *T. gondii* genome encodes a gene homolog to the human *inhibitor-2* gene. The resulting protein
8 sequence, TgI2, presents motifs that have been found in other I2 proteins to be important for
9 both interaction and regulation of the protein phosphatase 1 catalytic subunit. Surprisingly,
10 while most apicomplexan parasites only present two potential interaction motifs (Fig. 1B,
11 RVxF and HYNE), TgI2 protein sequence may encompass three potential motifs (SILK-like,
12 RVxF and HYNE). The presence of the SILK-like motif is supported by the alignment of the
13 TgI2 and HsI2 protein (Fig. 1A). However the sequence of the SILK-like motif in TgI2 does
14 not correspond to the motif consensus sequence [SG]ILK. The importance of the TgI2 SILK-
15 like motif is revealed by the ability of the TgI2 protein mutated for the two other motifs (RVxF
16 and HYNE) to inhibit the TgPP1 activity (Fig. 4G). The distribution of the motif across species
17 may argue for a later acquisition of the SILK domain in Opisthokonts. The presence of the
18 SILK-like domain in the distantly related eukaryote *T. gondii* but not in other Apicomplexan
19 parasites may argue for an independent acquisition and selection of this domain in *T. gondii*.
20 Other proteins in *T. gondii* display such a structure/function convergence to their mammalian
21 orthologs. For example toxofilin, a protein able to bind G-actin, was shown to exhibit two
22 sequences similar to sequences present in several G- and F-actin-binding proteins, while the
23 third appears to be specific to toxofilin (Jan *et al.*, 2007). Although the SILK-like domain of
24 TgI2 plays a minor role in the interaction with TgPP1, it has an important contribution in the
25 inhibitory activity of this protein (Fig. 4G). The SILK domain was shown to display similar

1 feature when mutated in the HsI2 protein, with a 500 fold decrease in activity after the Lysine
2 mutation (Park and DePaoli-Roach, 1994; Huang *et al.*, 1999). Similar to what was observed
3 for the HsI2, the SILK-like domain has a little role in the interaction with PP1c but is crucial
4 for its activity (Huang *et al.*, 1999). This apparent discrepancy between binding and inhibition
5 ability through the SILK domain of the I2 proteins may be due to the experimental condition
6 used to assess TgPP1 activity where binding affinity is not the limiting factor for TgPP1 activity
7 inhibition. The interactions at the SILK domain involve residues 12-17 (KGILK) of the HsI2
8 protein that bind primarily to a surface created by amino acids 50-59 in the PP1c subunit
9 (Hurley *et al.*, 2007). Structure-function studies have found that the two amino-acids (I¹⁴ and
10 L¹⁵) of the SILK domain are critical for the binding of surface formed by the PP1c E⁵⁴ and E⁵⁶
11 (Hurley *et al.*, 2007). Mutagenesis of either the I2 I¹⁴ and L¹⁵ or the PP1c E⁵⁴ and E⁵⁶ decreased
12 dramatically the PP1c inhibition (Huang *et al.*, 1999; Connor *et al.*, 2000). In the TgI2 protein,
13 the Isoleucine of the SILK domain is conserved while the Leucine is changed to a Glycine. This
14 exchange seems to be neutral for the capacity of the TgI2 SILK-like domain to inhibit the
15 TgPP1 activity, suggesting that there is a flexibility in this amino acid position for the activity
16 of the I2 protein. However, our mutagenesis experiments suggest a crucial involvement of the
17 conserved Lysine (K⁶) in the *T. gondii* ³VIGK⁶ (SILK) motif, since its mutation is sufficient to
18 abolish the SILK-mediated inhibitory activity of TgI2 on TgPP1 (Fig. 4). It has been suggested
19 that the Lysine in the GILK motif (K¹⁶) of the HsI2 protein is responsible for the proper
20 orientation of the protein when interacting with PP1c therefore enabling the interactions
21 contributed by the I¹⁴ and L¹⁵ (Hurley *et al.*, 2007). Our study confirms the importance of the
22 terminal Lysine of the SILK-like motif for the activity of TgI2.

23 The RVxF motif is shared by more than 70 % of the protein binding to PP1c (Hendrickx *et al.*,
24 2009). However, its consensus sequence [HKR][ACHKMNQRSTV][V][CHKNQRST][FW]
25 defined by Meiselbach *et al* (Meiselbach *et al.*, 2006). would not include the KSQKW sequence,

1 a well-established RVxF variant, present in the HsI2 sequence (Hurley *et al.*, 2007). This
2 sequence was nonetheless shown to be crucial for binding and activity of the HsI2 (Huang *et*
3 *al.*, 1999). The most remarkable change is the replacement of the Valine by a Glutamine which
4 was shown to bind to the PP1 (Hurley *et al.*, 2007) TgI2³³KHLTW³⁷ does match the consensus
5 sequence described earlier with the exception of the Valine that is changed to a Leucine. As for
6 the HsI2 or Pfl2 (where the Valine is replaced by an Isoleucine) and based on our mutagenesis
7 and binding assay, this change is not sufficient to prevent the binding of TgI2 to TgPP1.
8 Therefore, the consensus sequence for the RVxF motif should include other amino-acids at the
9 third position as suggested earlier (Hurley *et al.*, 2007). The RVxF motif of HsI2 was found to
10 bind at least the D¹⁶⁶ in HsPP1c within the RVxF binding groove (Hurley *et al.*, 2007).
11 Therefore, the SILK and RVxF motif are predicted to bind different region of the HsPP1c
12 protein. This would explain the complementarity of both SILK and RVxF motifs for the
13 inhibitory activity of TgI2. Since the RVxF groove on HsPP1c is the site of binding of multiple
14 proteins (Egloff *et al.*, 1997), the binding affinity may need to be greater than other site of the
15 I2 protein (e. g. the SILK motif) to accommodate the competition.

16 The HYNE motif of the HsI2 was found to directly interact with the HsPP1c catalytic domain
17 (Hurley *et al.*, 2007), therefore we anticipated that TgI2 HYNE mutants will show a defect in
18 their inhibitory activity. Moreover, this region of the sequence is highly conserved between
19 HsI2, Pfl2 and TgI2. In the assays presented here, the TgI2 protein mutated for the HYNE motif
20 behaved as the wild-type TgI2 protein. This suggests that the HYNE motif has only a minor
21 contribution in the activity of TgI2. Mutagenesis of the HYNE motif on the Pfl2 protein resulted
22 in a partial loss of the inhibitory activity of this protein (Fréville *et al.*, 2013), suggesting a
23 smaller contribution than the Pfl2 RVxF motif. However, site directed mutagenesis and deletion
24 of the HYNE motif of the HsI2 protein showed that it was not involved in inhibition of HsPP1c

1 (Huang *et al.*, 1999). Therefore, depending on the number of PP1 binding site on the protein,
2 the HYNE motif may not be relevant for the activity of I2 homologs.

3 TgI2 was found to localize at both the nuclear and cytoplasmic compartments as shown for the
4 PflI2 protein (Fréville *et al.*, 2013). TgPP1 localization is similarly distributed in both
5 compartments (Daher *et al.*, 2007) which suggests that they may interact in both. We found that
6 the PXTP motif is conserved in the TgI2 protein (⁵⁸PPTP⁶¹). This motif is known to be
7 phosphorylated during mitosis therefore increasing HslI2 localization to the centrosome (Leach
8 *et al.*, 2003). Phosphopeptides were mapped for the *T. gondii* proteome but no phosphopeptides
9 were recovered for this motif. Instead, two phosphorylated residues were uncovered for TgI2
10 (S67 and S77) in a region that is poorly conserved. Although, we cannot exclude that
11 phosphorylation of the PXTP motif occurs in *T. gondii*, there is no evidence for its participation
12 in TgI2 localization.

13 We show here that the TgI2 protein is a potent inhibitor of TgPP1 phosphatase activity. TgI2
14 SILK-like and RVxF motifs are critical for regulating the activity of TgPP1, a feature that is
15 common with the higher eukaryotes inhibitor-2 protein.

16

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1

2 **Fig. Legends**

3 **Fig. 1**

4 **A: Alignment of the *T. gondii*, *P. falciparum* and human inhibitor-2 proteins.**

5 Sequence alignment of the *T. gondii* (TGGT1_232760), *P. falciparum* (Pf3D7_0320000) and
6 human (NP_006232) I2 proteins. Similar amino-acids (aa) are highlighted in gray while
7 identical aa are highlighted in black. The SILK-like (blue), RVxF (red) and HYNE (green)
8 sequence are boxed.

9 **B: Analysis of the distribution of PP1 binding motifs among eukaryotes.**

10 Presence (+) or absence (-) of the respective motif is indicated for each species.

11 **Fig. 2: TgPP1 binding assay using different mutant TgI2 proteins.**

12 A: Western blot of the GST-pulled down proteins. The upper panel presents the signal given
13 after hybridization of the anti-His antibody, revealing the TgPP1-6His protein. The middle and
14 bottom panel represent the picture after hybridization of the same membrane using the anti-
15 GST antibody, revealing the GST tagged protein (TgI2 and the mutant). On the top of the Fig.
16 is indicated the lane number (2 to 10) as well as the protein that was used for the GST pull-
17 down. Lane 1 represents the input of the TgPP1 protein before the GST pull-down assay. The
18 three putative TgPP1 binding motifs of TgI2 (SILK-like, RVxF or HYNE) of the WT and each
19 mutant TgI2 protein are indicated at the top of the Fig.. Molecular weight markers are indicated
20 on the left side of the Fig.. Experiment was repeated three times, a representative experiment is
21 shown.

1 B: Quantification of the ratio of the TgPP1-6His signal over the GST tagged protein signal. The
2 signal for the His-tagged protein and GST-tagged protein was quantified using the densitometry
3 tool in the Image Lab software. The ratio of the His-tagged signal over the GST tagged signal
4 is represented here. On the bottom of the Fig. is indicated the protein that was used for the GST-
5 pull down. The three putative TgPP1 binding motifs of TgI2 (SILK-like, RVxF or HYNE) of
6 the WT and each mutant TgI2 protein are indicated at the bottom of the Fig.. A WT motif is
7 indicated in black. A mutated motif is indicated in red and crossed. Experiment was repeated
8 three times. Student's t-test comparing each mutant individually to the WT motifs, *** p-
9 value: $p < 0.001$; $\text{mean} \pm \text{s.d}$ (n=3).

10 **Fig. 3: TgPP1 is an active phosphatase responsive to okadaic acid inhibition.**

11 A: Graphical representation of the optic density at 405 nm measured after an hour of incubation
12 with TgPP1 (blue line) or commercial rabbit PP1 (green line) or TgPP1 without an essential
13 cofactor MnCl_2 (yellow line). The x-axis represents the quantity of PP1c used in the assay.
14 Experiment was repeated three times with duplicate measurement.

15 B: Graphical representation of the TgPP1 (blue line) or PfPP1 (yellow line) activity after
16 incubation with different concentrations of okadaic acid (indicated at the bottom of the Fig.).
17 Activity is represented as a % of the phosphatase activity measured without inhibitor.
18 Experiment was repeated three times with duplicate measurement.

19 **Fig. 4: TgPP1 activity is inhibited by TgI2 through different motifs.**

20 Graphical representation of the TgPP1 activity after incubation with different concentrations of
21 the TgI2 protein (indicated at the bottom of the Fig.). Activity is represented as a % of the
22 phosphatase activity measured without inhibitor. The three putative TgPP1 binding motifs of
23 TgI2 (SILK-like, RVxF or HYNE) of the WT and each mutant TgI2 protein are indicated at the
24 top of the Fig.. Experiment was repeated three times with duplicate measurement. Student's t-

1 test, *** p-value:p<0.001,** p-value:p<0.001; * p-value:p<0.01; ns: non-significant; mean±s.d
2 (n=3). A: WT TgI2 protein. B: TgI2 protein mutated at the three sites. C: TgI2 protein mutated
3 for the SILK-like motif. D: TgI2 protein mutated for the HYNE motif. E: TgI2 protein mutated
4 for the RVxF motif. F: TgI2 protein mutated for the SILK-like and RVxF motifs. G: TgI2
5 protein mutated for the RVxF and HYNE motifs. H: TgI2 protein mutated for the SILK-like
6 and HYNE motifs.

7 **Fig. 5: TgI2 is localized at the cytoplasmic and nuclear compartments.**

8 A: IFA analysis of the TgI2-HA (green) and TgENO2 (red) localization in the tachyzoite. DAPI
9 (blue) was used to stain DNA. The phase-contrast is also presented. The scale bar is indicated
10 at the bottom right of each picture.

11 B: Merge of the pictures presented in A with a quantitative fluorescence intensity plot. The Y-
12 axis of the plot represents the fluorescence intensities. The X-axis represents the distance from
13 the beginning to the end of the represented arrow.

14 C: IFA analysis of the TgI2-HA (green) and TgAlba 1 (red) localization in the tachyzoite. DAPI
15 (blue) was used to stain DNA. The phase-contrast is also presented. The scale bar is indicated
16 at the bottom right of each picture.

17 D: Merge of the pictures presented in C with a quantitative fluorescence intensity plot. The Y-
18 axis of the plot represents the fluorescence intensities. The X-axis represents the distance from
19 the beginning to the end of the represented arrow. The scale bar is indicated at the bottom right
20 of each picture.

21