Outside of well characterized model eukaryotes, relatively little is known about the translocons that transport proteins across the two membranes that surround the mitochondrion. Apicomplexans are a phylum of intracellular parasites that cause major diseases in humans and animals and are evolutionarily distant from model eukaryotes such as yeast. Apicomplexans harbor a mitochondrion that is essential for parasite survival and is a validated drug target. Here, we demonstrate that the apicomplexan Toxoplasma gondii harbors homologues of proteins from all the major mitochondrial protein translocons present in yeast, suggesting these arose early in eukaryotic evolution. We demonstrate that a T. gondii homologue of Tom22 (TgTom22), a central component of the translocon of the outer mitochondrial membrane (TOM) complex, is essential for parasite survival, mitochondrial protein import, and assembly of the TOM complex. We also identify and characterize a T. gondii homologue of Tom7 (TgTom7) that is important for parasite survival and mitochondrial protein import. Contrary to the role of Tom7 in yeast, TgTom7 is important for TOM complex stability, suggesting the role of this protein has diverged during eukaryotic evolution. Together, our study identifies conserved and modified features of mitochondrial protein import in apicomplexan parasites.

Mitochondria arose through the endosymbiotic acquisition of an α-proteobacterium, likely in a single event that occurred early in eukaryotic evolution (1, 2). Most known extant eukaryotes retain a mitochondrion or homologous organelle (3). Similar to their bacterial antecedents, most mitochondria retain a genome that encodes for some proteins. However, most proteins that function in the mitochondrion are nucleus-encoded and must post-translationally target across the two membranes that surround the mitochondrion. Proteins targeted to the mitochondrion contain either N-terminal presequences or internal targeting signals. These targeting signals interact with various protein translocases in the inner and outer membranes to ensure protein translocation across the mitochondrial membranes and into the appropriate compartment of the organelle.

Mitochondrial protein import is a core organellar function that was a critical early step in the evolution of mitochondria from α-proteobacteria (4). The last common ancestor of extant eukaryotes harbored a mitochondrion and also bore a functional mitochondrial protein import apparatus. However, it is becoming increasingly apparent that mitochondrial protein import machinery has diversified through the course of eukaryotic evolution.

Mitochondrial import has been most extensively studied in the yeast Saccharomyces cerevisiae (5). Translocation across the outer membrane is mediated by the translocon of the outer mitochondrial membrane (TOM) complex. The TOM complex represents the entry point into the organelle for most mitochondrial proteins. In yeast, the TOM complex includes the β-barrel protein Tom40, which forms the central pore of the complex (6). The TOM complex also includes the receptor proteins Tom20 and Tom70, three small Tom proteins (Tom5, Tom6, and Tom7) that function in regulating TOM complex assembly and function, and Tom22, a single-pass transmembrane protein that has several functions. The cytosolic N-terminal region of yeast Tom22 functions as a receptor domain that interacts with proteins as they enter from the cytosol (7). The transmembrane domain is critical for assembling the TOM complex into a higher order structure (8). The intermembrane space-localized C-terminal domain of Tom22 interacts with presequence-containing proteins as they pass through the TOM complex, an interaction that is critical for translocation of these proteins to the translocon of the inner membrane (9).

The outer mitochondrial membrane contains several β-barrel proteins. The targeting of these proteins involves translocation into the intermembrane space through the TOM complex, and subsequent insertion into the outer membrane by an outer membrane import mechanism called the sorting and assembly machinery

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The abbreviations used are: TOM, translocon of the outer mitochondrial membrane; SAM, sorting and assembly machinery; TIM, translocon of the inner mitochondrial membrane; PAM, presequence translocase-associated motor; MPP, mitochondrial processing peptidase; ATOM, archaic translocon of the outer mitochondrial membrane; ATC, anhydratetracycline; Shld1, Shield-1; LIC, ligation-independent cloning; BN-PAGE, blue native-PAGE; DDM, dodecyl maltoside; mDHFR, mouse dihydrofolate reductase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl) propane-1,3-diol; DD, destabilization domain; RFP, red fluorescent protein; 5'-RACE, 5'-rapid amplification of cDNA ends.
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(ATOM) complex, the central component of which is known as Sam50 (10).

The presequence translocase, also known as the translocase of the inner mitochondrial membrane 23 (TIM23) complex, translocates presequence-containing proteins across the inner membrane. Tim23 forms the pore through this membrane, whereas Tim50 functions as a receptor for proteins as they translocate from the TOM complex (11, 12). The presequence translocase can recruit a motor complex called the presequence-translocase associated motor (PAM) to drive ATP-dependent translocation of proteins into the mitochondrial matrix. The central component of the PAM complex is a mitochondrial Hsp70 that associates with J-domain proteins such as Pam18 (13). Upon translocation into the matrix, the presequence is proteolytically cleaved by a mitochondrial processing peptidase (MPP) to yield the mature protein (14), which can then fold and carry out its function.

Many mitochondrial proteins lack N-terminal presequences and instead harbor internal signals to direct them to the mitochondrion. Mitochondrial solute carrier proteins are inner membrane proteins that fall into this category. Mitochondrial carrier proteins enter the mitochondrion through the TOM complex and then interact with small Tim proteins such as Tim9 and Tim10 in the intermembrane space. These function to deliver carrier proteins to the TIM22 complex, which inserts carrier proteins into the inner membrane. The core component of this insertase is the protein Tim22 (15).

Although the mechanisms of mitochondrial import are well characterized in yeast and related organisms such as animals, this is less true of other eukaryotic lineages. The core components of the mitochondrial import machinery, including the TOM, TIM23, TIM22, PAM, and SAM complexes, are present in plants (16). There are, however, several major differences between plants and yeast, most notably features of the TOM complex. Plants lack homologues of the receptor proteins Tom70 and Tom20 and have evolved alternative receptor proteins (17, 18). Additionally, the plant Tom22 homologue is truncated at the N terminus and may not function as a presequence receptor (18, 19).

Another phylum where the molecular mechanisms of mitochondrial import have been functionally examined is the trypanosomatids, a group of parasites that include Trypanosoma brucei, the causative agent of African sleeping sickness. The outer membrane translocase of trypanosomatids is termed the archaic translocase of the outer mitochondrial membrane (ATOM) complex and appears different from the TOM complex in yeast. The central component of the ATOM complex is a β-barrel protein called Atom40 (20). There is debate as to whether Atom40 is homologous to Tom40 (21, 22). Studies have identified other ATOM protein components, including Atom69, Atom46, Atom36, Atom14, Atom12, and Atom11 (23, 24). These components lack clear homologues in yeast, although hidden Markov model analysis suggests that Atom14 is homologous to Tom22 (21). Trypanosomatid genomes harbor a single TIM translocase protein homologue, which is essential for import of presequence-containing proteins (25, 26).

Apicomplexans are a phylum of intracellular parasites. They include important human parasites such as the Plasmodium species, the causative agents of malaria, and Toxoplasma gondii, a parasite that causes congenital disease in unborn children and encephalitis in immunocompromised people. Most apicomplexans harbor a mitochondrion, which is the target of major and emerging anti-apicomplexan drugs such as atovaquone, endochin-like quinolones, and triazolopyrimidines (27–29). Studies of the mitochondrial protein import machinery of apicomplexans have been limited to comparative genomic approaches (30–32). These have identified putative homologues of the core components of mitochondrial import, including members of the TOM, TIM23, TIM22, PAM, and SAM complexes.

The TOM complex of apicomplexans appears divergent from that found in yeast. Apicomplexan genomes lack identifiable homologues to TOM complex receptors such as Tom70 and Tom20. Like plants, the N terminus of Plasmodium Tom22 appears truncated (19). Additionally, these comparative approaches have not identified Tom7 homologues in apicomplexan genomes, making apicomplexans one of the few lineages where a TOM complex is present that appears to lack Tom7 (19, 21).

The last common ancestor of apicomplexans and yeast contained a mitochondrion and must have had a means of targeting nucleus-encoded proteins into this organelle. In this paper, we ask the following. What features of this mitochondrial import apparatus have been conserved from the last common ancestor? What new features have arisen? We use T. gondii as our model system to perform the first broad functional analysis of the mitochondrial protein import machinery in Apicomplexa. In particular, we focus on the TOM complex and assay the role of two TOM complex components in mitochondrial import and TOM complex biogenesis. These studies provide insights into conserved and novel functions of mitochondrial import machinery across their evolution, and they provide a platform for future studies of mitochondrial import in an evolutionarily interesting and medically important group of organisms.

Results

Identity and Localization of Putative Mitochondrial Import Proteins in T. gondii—To identify candidate mitochondrial import proteins, we performed Basic Local Alignment Search Tool (BLAST) searches, querying the T. gondii genome with characterized mitochondrial import proteins from the yeast S. cerevisiae, the plant Arabidopsis thaliana, and the trypanosomatid T. brucei. Matches were confirmed through reciprocal BLAST searches against the T. gondii genome. The results of these searches are summarized in supplemental Table S1. We identified homologues of major subunits from each of the translocases and insertases involved in protein import into the mitochondrion. These include homologues of Tom40, Sam50, Tim23, Tim17, Tim50, Hsp70, Mge1, Tim44, Pam18, Tim22, and a host of small Tims. We also identified a T. gondii homologue of Oxa1, a protein that functions in the insertion of inner membrane proteins encoded on the mitochondrial genome (supplemental Table S1) (33).
Some notable proteins lacked detectable homologues in *T. gondii*. This was particularly the case for the TOM complex, for which we were unable to identify candidates for receptor proteins (e.g. Tom70 and Tom20) or small Tom proteins (e.g. Tom5 and Tom7). Additionally, we were unable to identify components of the SAM and carrier insertase complexes beyond the central Sam50 and Tim22 subunits. We were also unable to identify a homologue of MIA40, a protein with a central role in the import of small Tim proteins into the intermembrane space (34).

To determine whether these candidate mitochondrial import proteins localized to the mitochondrion, we epitope-tagged select members of each protein complex. We first tagged *Tg*Tom40, the putative import pore in the outer membrane, and demonstrated co-localization with mitochondrially targeted *RFP* (Fig. 1A). Additionally, we generated polyclonal rabbit antibodies against *Tg*Tom40. Immunofluorescence assays using this antibody supported the mitochondrial localization of *Tg*Tom40 (Fig. 1B). Analysis by Western blotting revealed that *Tg*Tom40 is a protein with a mass of 48 kDa (Fig. 1C). Through co-localization with either *Tg*Tom40 or mitochondrial RFP, and associated Western blotting, we demonstrated mitochondrial localization and protein expression for the following proteins: *Tg*Sam50 (Fig. 1, D and E), consistent with the mitochondrial localization of this protein reported in a recent study (35); *Tg*Tim22 (Fig. 1, F and G); *Tg*Tim23 (Fig. 1, H and I); *Tg*Tim50 (Fig. 1, J and K); and *Tg*MPPα (Fig. 1, L and M). We were unable to generate a parasite strain that stably expressed epitope-tagged *Tg*Pam18. Instead, we transiently overexpressed c-Myc-tagged *Tg*Pam18 in *T. gondii* parasites and performed an immunofluorescence assay. This revealed mitochondrial localization of *Tg*Pam18 (Fig. 1N). Taken together, these data indicate that *T. gondii* homologues of each of the major mitochondrial protein translocases and insertases localize to the mitochondrion (Fig. 1O).

Localization of the Tom22 Homologue of *T. gondii*—Our bioinformatics survey (supplemental Table S1) identified a *T. gondii* homologue of Tom22 that we called *Tg*Tom22. We performed an alignment of *Tg*Tom22 with Tom22 homologues from *Plasmodium falciparum*, yeast, humans, and plants (Fig. 2A). This alignment revealed that the N terminus of *Tg*Tom22 is truncated compared with yeast and human Tom22, as noted previously for Tom22 homologues in plants and *Plasmodium* (19). We identified two predicted transmembrane domains in *Tg*Tom22 (Fig. 2A). Sequence conservation among putative Tom22 homologues is generally poor, with only three identical residues found in all the proteins presented in the alignment, all three of which are found in the predicted transmembrane domain.
To localize \( TgTom22 \), and to facilitate subsequent functional analysis of this protein, we introduced an N-terminal hemagglutinin (HA) epitope-tagged \( TgTom22 \) into the TATi strain of \( T. gondii \). This exogenous version of \( TgTom22 \) was placed under the control of an anhydrotetracycline (ATc)-regulated promoter. We termed the resultant strain \( rTom22/eTom22 \), to indicate the presence of both regulatable and endogenous copies of \( TgTom22 \). We then knocked out the native \( Tom22 \) locus, verifying successful knock-out by PCR analysis (Fig. 3, A and B).

The resultant strain was termed \( rTom22/H9004tom22 \) and expressed only the ATc-regulatable HA-tagged \( TgTom22 \). Immunofluorescence analysis of this strain revealed that HA-Tom22 co-localizes with \( TgTom40 \), indicating that \( TgTom22 \) is a mitochondrial protein (Fig. 2B). Western blotting revealed that HA-Tom22 is 10 kDa in mass, close to the predicted mass of 12 kDa for the HA-tagged protein. To test whether \( TgTom22 \) is a transmembrane protein, we extracted \( T. gondii \) parasites in the \( rTom22/H9004tom22 \) strain with either the detergent Triton X-100 or sodium carbonate (\( Na_2CO_3 \)) at pH 11.5. HA-Tom22 partitions into the pellet phase of the \( Na_2CO_3 \) extraction, much like the integral mitochondrial membrane protein \( Tom40 \) and unlike the soluble mitochondrial matrix protein ATP synthase \( \beta \) subunit, which partitions predominantly into the supernatant (S/N) phase.

Characterization of the TOM Complex of \( T. gondii \)—As a first step to characterizing the TOM complex of \( T. gondii \), we performed blue native-PAGE (BN-PAGE) on \( rTom22/\Delta tom22 \) strain \( T. gondii \) parasites, probing Western blottings with the \( TgTom40 \) antibody. When solubilized in either 0.5 or 1% digitonin, \( TgTom40 \) localizes to a protein complex of around 190 kDa. This is similar to the predicted integral membrane protein \( Tom40 \) and distinct from the \( \beta \)-subunit of ATP synthase, a predicted soluble protein. We conclude that \( TgTom22 \) is a mitochondrial integral membrane protein.
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A. Schematic describing knock-out of the native TgTom22 gene. The native TgTom22 locus (orange) was replaced with a chloramphenicol acetyltransferase (CAT)-selectable marker (blue), in a background strain expressing an ATc-regulatable copy of TgTom22 (data not shown). B. PCR screen of 12 parasite clones transfected with the TgTom22 knock-out construct with primers 21 and 22. The primer pair will produce a band of 0.8 kb if the native gene is present and a band of 1.6 kb when the regulatable copy of TgTom22 is present. Clones 3 and 5–11 lack the native gene, indicating successful knock-out of the TgTom22 native locus in these parasites. C. Schematic describing replacement of the native TgTom7 promoter with an ATc-regulated promoter (t7s4) and 5’ HA tag. The TATi/Δku80 parasite strain was transfected with a vector containing 5’ and 3’ flanks of the TgTom7 gene, the t7s4 ATc-regulated promoter, a 5’ HA tag, and a pyrimethamine-resistant T. gondii DHFR-selectable marker. D. PCR screen of six parasite clones transfected with the promoter replacement vector and screened with primer pairs 31 and 32 to detect the native TgTom7 locus (top) or primer pairs 32 and 33 to detect the modified locus (bottom). DNA from wild-type (WT) TATi/Δku80 parasites was used as a control. Clones 1, 3, and 4 lack the band corresponding to the presence of the native gene but harbor the band corresponding to the modified locus, indicating successful promoter replacement at the TgTom7 locus in these parasites.

When solubilized in 1% dodecyl maltoside or 1% Triton X-100, TgTom40 localizes to a slightly smaller complex of around 325 kDa. The TgTom40 complex appeared insoluble in octyl β-D-glucopyranoside. We conclude that the TgTom40 protein is part of an ~400-kDa protein complex in the mitochondrion of T. gondii, which is similar in mass to characterized TOM complexes measured by BN-PAGE in yeast (36). We refer to this complex as the T. gondii TOM complex.

We hypothesized that TgTom22 localizes to the TOM complex, whereas TgSam50 localizes to a separate complex. To test this, we expressed a c-Myc epitope-tagged TgSam50 in the rTom22/Δtom22 cell line. We initially performed one-dimensional BN-PAGE and were unable to identify either HA-Tom22 or TgSam50-Myc protein, likely because the epitope tags on these proteins were inaccessible to antibodies when in the complete complex.4 We therefore performed two-dimensional BN-PAGE, separating protein complexes in the second dimension using SDS-PAGE. These studies revealed that TgTom40 is predominantly found in a 400-kDa complex that corresponds in mass to the 400-kDa complex containing TgTom40. TgSam50-c-Myc localizes in complexes of 200 and 350 kDa (Fig. 4B).

To further test the composition of the T. gondii TOM complex, we performed co-immunoprecipitation experiments on the TgSam50-c-Myc/rTom22/Δtom22 cell line. We solubilized T. gondii proteins in 0.5% digitonin and immunoprecipitated TgTom40 and all interacting proteins using anti-TgTom40 antibodies. HA-Tom22, but not TgSam50, co-immunoprecipitated with TgTom40 antibodies (Fig. 4C).

Next, we performed pulldowns of HA-Tom22-interacting proteins using anti-HA antibodies. These studies revealed that a large fraction of TgTom40, but not TgSam50, associated with HA-Tom22 (Fig. 4C). Together, these results indicate that TgTom40 and TgTom22 form part of a core 400-kDa TOM complex that does not include TgSam50.

TgTom22 Is Essential for Parasite Growth—Having generated an ATc-regulatable TgTom22 cell line, we sought to determine whether TgTom22 was essential for parasite survival. We first measured knockdown of HA-Tom22 by growing parasites for 0–2 days in ATc. This revealed that HA-Tom22 protein levels are much reduced 1 day after the addition of ATc and are undetectable after 2 days (Fig. 5A).

To determine whether TgTom22 is required for growth of T. gondii parasites, we introduced a tandem tomato red fluorescence protein into the rTom22/Δtom22 cell line. This allows daily quantification of parasite growth as a function of well fluorescence in a 96-well plate (37, 38). rTom22/Δtom22 parasites cultured in the presence of ATc showed no detectable growth.

4 G. van Dooren, unpublished observations.
across the 8-day experiment (Fig. 5B). These data indicate that TgTom22 is critical for parasite growth. To determine whether the observed defect in growth in the presence of ATc is dependent on TgTom22, we complemented the rTom22/H9004tom22 cell line with constitutively expressed TgTom22, producing the strain we termed rTom22/H9004tom22/Tom22WT. Parasite growth in this strain was equivalent with or without ATc (Fig. 5C), consistent with the observed growth effect in mutant parasites resulting solely from loss of TgTom22.

TgTom22 Is Essential for Mitochondrial Protein Import—We next wanted to determine the function(s) of TgTom22. We hypothesized that TgTom22 plays a role in mitochondrial protein import. Proteins targeted to the matrix of mitochondria harbor an N-terminal presequence that is removed upon entry into the matrix. Presequence cleavage therefore serves as a robust measure for mitochondrial protein import. To monitor this, we fused the presequence leader of the mitochondrial protein TgHsp60 (Hsp60\textsubscript{Tg}) to a c-Myc-tagged mouse DHFR (mDHFR) reporter protein (mDHFR has been used extensively in studies of mitochondrial import in other systems) and introduced this Hsp60\textsubscript{Tg}-mDHFR-cMyc protein construct into rTom22/\Delta tom22/Tom22WT parasites. We grew parasites for 0–3 days on ATc and measured the abundance of a range of proteins by Western blotting. As shown previously, HA-Tom22 was undetectable after 2 days on ATc (Fig. 6A). In the presence of HA-Tom22, we observed a single Hsp60\textsubscript{Tg}-mDHFR-cMyc band that corresponds in mass to the mature (processed) form of this protein, where the presequence has been removed (Fig. 6A). Upon loss of HA-Tom22, we noted an accumulation of the precursor form of Hsp60\textsubscript{Tg}-mDHFR-cMyc, as well as the precursor form of the native TgHsp60 protein (Fig. 6A). Notably, Tom40 levels remain unchanged with the loss of HA-Tom22.

As a more sensitive measure of mitochondrial protein import, we performed a radioactive labeling experiment. We incubated rTom22/\Delta tom22 parasites expressing Hsp60\textsubscript{Tg}-mDHFR-cMyc for 10, 30, and 60 min in medium containing \[^{35}\text{S}]\text{methionine and }[^{35}\text{S}]\text{cysteine. We then performed immunoprecipitations to purify Hsp60\textsubscript{Tg}-mDHFR-cMyc protein and separated proteins by SDS-PAGE. In the absence of ATc, we observed two protein bands, corresponding in mass to the precursor and mature forms of Hsp60\textsubscript{Tg}-mDHFR-cMyc (Fig. 6B). Only a small amount of mature Hsp60\textsubscript{Tg}-mDHFR-cMyc was present at the 10-min time point in the absence of ATc, but this increased upon longer incubation in the radioactive medium, consistent with protein synthesis followed by precursor processing upon mitochondrial import. In contrast, we observed only the precursor form of Hsp60\textsubscript{Tg}-mDHFR-cMyc in parasites grown for 2 days on ATc, indicative of an absence of mitochondrial protein import in these parasites. We conclude that TgTom22 is critical for mitochondrial protein import.

TgTom22 Is Critical for TOM Complex Assembly—We next sought to determine the functional roles of TgTom22. We first asked whether TgTom22 has a role in TOM complex assembly. We performed BN-PAGE on proteins extracted from rTom22/\Delta tom22 parasites grown for 0–3 days on ATc and measured the abundance of a range of proteins by Western blotting. As shown previously, HA-Tom22 was undetectable after 2 days on ATc (Fig. 6A). In the presence of HA-Tom22, we observed a single Hsp60\textsubscript{Tg}-mDHFR-cMyc band that corresponds in mass to the mature (processed) form of this protein, where the presequence has been removed (Fig. 6A). Upon loss of HA-Tom22, we noted an accumulation of the precursor form of Hsp60\textsubscript{Tg}-mDHFR-cMyc, as well as the precursor form of the native TgHsp60 protein (Fig. 6A). Notably, Tom40 levels remain unchanged with the loss of HA-Tom22.

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into rTom22/Δtom22 parasites to derive the parasite strain rTom22/Δtom22/DD-Tom22WT. The stability of DD-tagged proteins can be controlled through the addition of the small molecule Shld1, allowing for rapid control of protein levels (39). We monitored the growth of both rTom22/Δtom22 and rTom22/Δtom22/DD-Tom22WT parasites using the previously described fluorescence growth assay, incubating parasites either in the absence of ATc and Shld1, in the presence of ATc and absence of Shld1, or in the presence of both ATc and Shld1 (to turn off expression of regulatable HA-Tom22 and to stabilize DD-Tom22WT). Growth of rTom22/Δtom22 parasites was negligible in the presence of ATc, regardless of the presence of Shld1 (Fig. 6F). In the rTom22/Δtom22/DD-Tom22WT parasite strain, however, parasite growth in the presence of ATc was restored to near wild-type levels upon the addition of Shld1 (Fig. 6F). This indicates that DD-Tom22WT can complement the rTom22/Δtom22 mutant.

We grew rTom22/Δtom22/DD-Tom22WT parasites for 0 or 48 h in ATc. For the parasites grown for 48 h in ATc, we added Shld1 0, 3, 6, 18, or 48 h before harvesting. We then performed BN-PAGE on proteins extracted from these cell lines. In the absence of ATc, TgTom40 is found in the ~400-kDa TOM complex, whereas the addition of ATc disrupts the 400-kDa complex in the absence of Shld1 (0 h; Fig. 6G). Three hours after the addition of Shld1, we see re-formation of the 400-kDa TOM complex, and the abundance of the TOM complex increases with increased time on Shld1 (Fig. 6G). Three and 6 h after the addition of Shld1, we observed some TgTom40 in smaller protein complexes of ~150 and ~300 kDa, which may represent intermediates in TOM complex assembly. We conclude that TgTom22 has a critical, and likely direct, role in TOM complex assembly.

Apicomplexan Parasites Have a Tom7 Homologue That Is Important for TOM Complex Assembly and Parasite Growth—T. gondii harbors a TOM complex of around 400 kDa that contains TgTom40 and TgTom22. We reasoned that other proteins must be part of this complex as well. Previous bioinformatics approaches have been unable to identify homologues of small TOM complex proteins such as Tom7 in apicomplexans, even though these are present in the genomes of many other eukaryotes (19, 21). Given the limited primary sequence conservation, we reasoned that homology searches might be insufficient to identify Tom7 homologues in these parasites. From an alignment of Tom7s from multiple organisms, we noted that the conserved feature of all of these is a G(x)3P(x)1G motif in the single transmembrane domain. We searched the genome of the apicomplexan parasite P. falciparum for predicted proteins that harbor this motif. We then screened these candidates further for proteins of less than 15 kDa that harbored a single transmembrane domain. This produced one candidate, a hypothetical protein annotated as PF3D7_0823700. Using this as a query sequence, we identified a homologous protein encoded in the T. gondii genome (TGME49_210255) and termed this TgTom7. We performed 5'-rapid amplification of cDNA ends (5'-RACE) to identify the open reading frame and establish its sequence.

We constructed an alignment of the putative TgTom7 protein with Tom7 homologues in other organisms, revealing the
presence of the G(X)\textsubscript{2}P(X)\textsubscript{5}G in the transmembrane domain, but little further conservation (Fig. 7A). The TgTom7 open reading frame contains two potential start codons. Alignments of TgTom7 sequence with the homologous protein in Neospora caninum, a close relative of T. gondii, revealed no conservation upstream of the second start codon (Fig. 7A). Furthermore, subsequent analysis revealed that the longer form of TgTom7 exhibits similar localization and mutant phenotypes to the short form, suggesting that the region upstream of the second start codon is dispensable for function. Together, these data suggest that the second codon may represent the true start codon in this gene.

To localize TgTom7, we fused a region encoding a 3XHA tag to the 5' end of the open reading frame to enable detection of the resultant protein (which we termed HA\textsubscript{3}Tom7). To facilitate subsequent functional analysis, we also replaced the
native promoter of this gene with an ATc-regulated promoter in the TATi/Δku80 parasite strain (41) to generate the parasite strain we termed TATi/Δku80/ku80/rTom7. We confirmed successful integration of the 5′-HA tag and ATc-regulated promoter through PCR analysis (Fig. 3, C and D). We performed an immunofluorescence assay demonstrating co-localization of HA-tagged version of the short isoform of TgTom7 (HA3-Tom7; green) with the mitochondrial marker Tom40 (red). Scale bar, 2 μm. C, Western blotting analysis of HA3-Tom7, probed with anti-HA antibodies, reveals a protein of 11 kDa. D, Western blotting of proteins extracted from the TATi/Δku80/rTom7 parasite strain and separated by BN-PAGE in a first dimension and SDS-PAGE in a second dimension. Blots were probed with anti-Tom40 antibodies (top) and anti-HA antibodies to detect HA3-Tom7 protein (bottom). E, Western blotting of parasite proteins extracted from TATi/Δku80/rTom7 strain parasites in 0.5% digitonin, immunoprecipitated with anti-Tom40 antibodies, and separated by SDS-PAGE. Blots were probed with anti-Tom40 antibodies (top) and anti-HA antibodies (bottom). Total proteins, unbound proteins, and bound proteins were loaded for each experiment, and each lane contains proteins extracted from an equivalent number of parasites. The heavy chain of anti-Tom40 antibodies was detectable in the anti-Tom40 Western blotting.

We performed a co-immunoprecipitation with antibodies against TgTom40. Most of the HA3-Tom7 protein appeared in the bound fraction (Fig. 7E), indicating that HA3-Tom7 is part of a complex with TgTom40. We attempted the reciprocal experiment of co-immunoprecipitating with anti-HA-coupled beads. We were unable to purify either HA3-Tom7 or TgTom40, suggesting that the HA tag on TgTom7 may be hidden from antibodies within the complex.

We next asked whether TgTom7 was important for parasite growth. First, we determined whether the addition of ATc resulted in knockdown of the HA3-Tom7 protein. We cultured parasites in ATc for 0–3 days and measured protein abundance. HA3-Tom7 protein was undetectable after 2 days growth on ATc (Fig. 8A). To test whether TgTom7 is important for parasite growth, we introduced a tandem tomato red fluorescent protein into the rTom22/ΔTom7 strain parasites. These revealed that TATi/Δku80/rTom7 strain parasites alone exhibited growth impairment in the presence of ATc (Fig. 8, B and C).

FIGURE 7. TgTom7 is a mitochondrial protein and a constituent of the TOM complex. A, multiple protein sequence alignment of the long and short isoforms of TgTom7 with homologues from N. caninum (NcTom7), P. falciparum (PfTom7), S. cerevisiae (ScTom7), A. thaliana (AtTom7-1), and humans (HsTom7). Transmembrane domains of each protein (predicted using TMHMM) are highlighted by a box. B, Immunofluorescence assay demonstrating co-localization of HA-tagged version of the short isoform of TgTom7 (HA3-Tom7; green) with the mitochondrial marker Tom40 (red). Scale bar, 2 μm. C, Western blotting analysis of HA3-Tom7, probed with anti-HA antibodies, reveals a protein of 11 kDa. D, Western blotting of proteins extracted from the TATi/Δku80/rTom7 parasite strain and separated by BN-PAGE in a first dimension and SDS-PAGE in a second dimension. Blots were probed with anti-Tom40 antibodies (top) and anti-HA antibodies to detect HA3-Tom7 protein (bottom). E, Western blotting of parasite proteins extracted from TATi/Δku80/rTom7 strain parasites in 0.5% digitonin, immunoprecipitated with anti-Tom40 antibodies, and separated by SDS-PAGE. Blots were probed with anti-Tom40 antibodies (top) and anti-HA antibodies (bottom). Total proteins, unbound proteins, and bound proteins were loaded for each experiment, and each lane contains proteins extracted from an equivalent number of parasites. The heavy chain of anti-Tom40 antibodies was detectable in the anti-Tom40 Western blotting.
To ascertain whether this growth phenotype resulted solely from down-regulation of \( \text{TgTom7} \) expression, we complemented the TATi/\( \Delta \text{kku80}/\text{rTom7} \) strain with \( \text{TgTom7} \) expressed from a constitutive promoter to produce a cell line termed TATi/\( \Delta \text{kku80}/\text{rTom7}/\text{Tom7WT} \). We then introduced a tandem tomato red fluorescent protein and performed fluorescent growth assays. This revealed that this complemented strain was fully restored in growth (Fig. 8D), suggesting that the growth defect we observed results entirely from the decrease in \( \text{TgTom7} \) expression.

We next wanted to determine whether \( \text{TgTom7} \) has a role in mitochondrial protein import. Attempts to generate a TATi/\( \Delta \text{kku80}/\text{rTom7} \) strain expressing \( \text{TgTom7} \) from a constitutive promoter to produce a cell line termed TATi/\( \Delta \text{kku80}/\text{rTom7}/\text{Tom7WT} \) were unsuccessful. Instead, we performed Western blotting on TATi/\( \Delta \text{kku80}/\text{rTom7} \) parasites grown for 0–3 days in ATc and probed with antibodies against mitochondrial Hsp60. When Tom7 was present (day 0), we observe a single \( \text{TgHsp60} \) protein species that corresponds to the mature mitochondrially localized \( \text{TgHsp60} \) (Fig. 8A). Two days after the addition of ATc and concomitant with \( \text{TgTom7} \) knockdown, we observed the appearance of a higher molecular mass species, corresponding to presequence-containing \( \text{TgHsp60} \). This band increased in abundance after 3 days on ATc (Fig. 8A). Notably, the abundance of \( \text{TgTom40} \) remains unchanged upon the addition of ATc. These data are consistent with a role for \( \text{TgTom7} \) in mitochondrial protein import that does not depend on regulating the stability or turnover of \( \text{TgTom40} \).

Given the role of \( \text{TgTom22} \) in TOM complex assembly, we wanted to determine whether \( \text{TgTom7} \) was also important for this process. We performed BN-PAGE to measure TOM complex assembly upon HA3-Tom7 knockdown. These studies revealed loss of the TOM complex concomitant with HA3-Tom7 knockdown (Fig. 8E). We performed two-dimensional
We demonstrate that loss of TgTom22 leads to defects in maturation of mitochondrial matrix proteins, consistent with a role for TgTom22 in mitochondrial protein import. Studies from yeast have identified a key role for Tom22 in TOM complex assembly (8). Our data suggest TgTom22 has a direct role in TOM complex assembly, mirroring the role of Tom22 in yeast. In yeast, loss of Tom22 results in disassembly of the TOM complex to form an ∼100-kDa complex. In T. gondii, TgTom22 knockdown leads to the formation of two major protein complexes of 200 and 300 kDa (Fig. 6D). It is unclear whether these are simply disassembled TOM complexes or precursors of the TOM complex, although it is interesting to note that SAM50 forms complexes of ∼200 kDa (Fig. 4B), suggesting the smaller of the complexes could be a SAM-TOM complex intermediate. Also unclear is whether these depleted TgTom40-containing complexes can function in import. Regardless, the conserved role of Tom22 in the TOM complex assembly between yeast and apicomplexans suggests that one role of Tom22 in the last common ancestor of these lineages was assembly of the TOM complex. This points to an ancient and conserved role for Tom22 in TOM complex assembly.

In yeast, Tom7 is a non-essential protein that negatively regulates TOM complex assembly (48, 49). Loss of Tom7 in yeast therefore promotes TOM complex assembly. Previously, apicomplexans were thought to be one of only a few TOM complex-containing eukaryotic lineages that lacked a Tom7 homologue (19). We revisited this and were able to identify a candidate Tom7 homologue in both T. gondii and P. falciparum. We could demonstrate that TgTom7 was indeed a component of the core TOM complex in these parasites and important for mitochondrial protein import.

In contrast to yeast, loss of TgTom7 leads to a growth defect in T. gondii parasites and impairment of TOM complex assembly. We observe TOM complex dissociation concomitantly with TgTom7 knockdown, suggesting the two processes are directly linked. Nevertheless, we were unable to generate a tightly regulated DD-tagged TgTom7 cell line, which precluded a more robust test of this hypothesis. Notably, although knockdown of both TgTom7 and TgTom22 results in dissociation of the TOM complex, loss of TgTom22 leads to a considerably stronger growth defect (cf. Figs. 5B and 8C), and a qualitatively more severe defect in mitochondrial protein import (cf. Figs. 6A and 8A). This suggests that protein import is not entirely ablated upon TOM complex disassembly. It also suggests that, in addition to its role in TOM complex assembly, TgTom22 has other roles in mitochondrial protein import. The C-terminal intermembrane space domain of Tom22 in other organisms is critical for translocating proteins from the TOM complex to the presequence translocase (9), and it is conceivable that TgTom22 has a role in this process.
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| No. | Primer name                      | Primer sequence                                                                 |
|-----|---------------------------------|---------------------------------------------------------------------------------|
| 1   | Tom40 ORF 3' rep fwd            | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 2   | Tom40 ORF 3' rep rvs            | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 3   | Tom40 ORF fwd                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 4   | Tom40 ORF rvs                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 5   | Tim22 3' rep fwd                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 6   | Tim22 3' rep rvs                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 7   | Tim23 3' rep fwd                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 8   | Tim23 3' rep rvs                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 9   | Tim50 3' rep fwd                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 10  | Tim50 3' rep rvs                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 11  | Tom18 ORF fwd                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 12  | Tom18 ORF rvs                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 13  | MPPB 3' rep fwd                 | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 14  | MPPB 3' rep rvs                 | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 15  | Tom22 ORF fwd                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 16  | Tom22 ORF rvs                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 17  | Tom22 5' flank fwd              | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 18  | Tom22 5' flank rvs              | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 19  | Tom22 3' flank fwd              | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 20  | Tom22 3' flank rvs              | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 21  | Tom22 screen fwd                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 22  | Tom22 screen rvs                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 23  | Tom22 comp fwd                  | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 24  | Tom22 comp rvs                  | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 25  | Tom5’ 5’-RACE                    | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 26  | Tom7 5’-RACE nested             | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 27  | Tom7 ORF 3' flank fwd           | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 28  | Tom7 ORF 3' flank rvs           | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 29  | Tom7 5' flank fwd               | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 30  | Tom7 5' flank rvs               | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 31  | Tom7 screen fwd                 | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 32  | Tom7 screen rvs                 | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 33  | Tim14 screen fwd                | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 34  | Tom7 comp fwd                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 35  | Tom7 comp rvs                   | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 36  | Tom40 Ab fwd                    | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|
| 37  | Tom40 Ab rvs                    | 5’-TACCTCCCAATCGAGTTTAAAGTTGACGTAACAGTCGAGGTAGCACGACGCTTCAGCCAGG|

Apicomplexan Tom7 is the first non-opisthokont Tom7 that has been functionally characterized. The contrasting role of Tom7 between apicomplexans and opisthokonts suggests that, even though it is conserved as part of the “core” TOM complex, the functions of Tom7 have diverged substantially during eukaryotic evolution. This is in contrast to our findings with Tom22, where the role of Tom22 in TOM complex assembly appears conserved. From our functional analyses, we can conclude that the TOM complex of apicomplexan parasites contains both conserved and unique features when compared with the TOM complex of yeast.

**Experimental Procedures**

**Parasite Culture and Growth Assays**—Parasites were grown in human foreskin fibroblasts using Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum and antibiotics. Strains used included RH, TATi, Δku80, and TATi/Δku80 (41, 50, 51). All parasite strains described in this work were cloned by limiting dilution. Where required, we added ATC at a final concentration of 0.5 µg/ml and Shld1 at a final concentration of 0.75 µM. Fluorescence growth assays were performed in optical bottom 96-well plates, as described previously (37, 38), and read using a FLUOstar Optima fluorescence plate reader (BMG Labtech).

**Plasmid Construction**—To determine the localization of the candidate mitochondrial import-related proteins, we introduced epitope tags at the 3’ end of either the endogenous locus of the genes encoding these proteins or of the open reading frame of candidate genes expressed from constitutive promoters. To localize TgTom40, we amplified the 3’ region with primers 1 and 2 (Table 1) and inserted this into the vector pHA3.LIC.DHFR (a kind gift from Michael White, University of South Florida) by ligation-independent cloning (LIC), as described previously (50), before linearization with EcoRV, transfection into Δku80 strain parasites, and selection on pyrimethamine as described (52). To localize TgSam50, we amplified the open reading frame with primers 3 and 4, digested the resulting product with BglII and AvrII, ligated into the vector pBTM3, transfected into parasites, and selected on phleomycin as described (54). To localize TgTim22, we amplified the 3’ region with primers 5 and 6, digested the resulting product with BglII and AvrII, ligated into pH4 vector, a modified version of the pgCM3 vector described previously (55) that has a selectable marker for mycophenolic acid selection. The vector was linearized with AflIII, transfected into parasites, and selected on mycophenolic acid as described (56). To localize TgTim23, we amplified the 3’ region with primers 7 and 8, digested the resulting product with BglII and AvrII, and ligated into the pH4 vector a modified version of the pH4 vector that contains a phleomycin resistance marker. This vector was linearized with NcoI, transfected into parasites, and selected on phleomycin. To localize TgTim50, we amplified the 3’ region with primers 9 and 10, inserted the product into the vector.
pTy.LIC.DHFR, a modified version of pHA<sub>3</sub>.LIC.DHFR where the HA tag has been replaced by a 1xTy1 tag, through LIC. The resulting vector was digested with NsiI, transfected into parasites, and selected on pyrimethamine. To localize TgPam18, we amplified the open reading frame with primers 11 and 12, digested the resulting product with BglII and AvrII, and ligated into the vector pBTM<sub>1</sub>. We transfected the resulting vector into parasites and observed transient expression after 1 day. To localize TgMPP<sub>α</sub>, we amplified the 3’ region with primers 13 and 14, inserted this into pHA<sub>3</sub>.LIC.DHFR by LIC, linearized with NcoI, transfected into parasites, and selected on pyrimethamine.

To generate an ATc-regulated knockdown strain of TgTom22, we amplified the TgTom22 open reading frame with primers 15 and 16, digested the resulting product with XmaI and AatII, and ligated into the equivalent sites of the pDT7s4H vector (56). This vector was transfected into TATi strain parasites and selected on pyrimethamine. To knock-out the native TgTom22 gene, we amplified a region upstream of the TgTom22 start codon with primers 17 and 18, digested the resulting product with SpeI and BglII, and ligated into the equivalent sites on the vector pTCY (38). We then amplified a region downstream of the TgTom22 stop codon using primers 19 and 20, digested the resulting product with Sall and Apal, and ligated into equivalent sites of the pTCY vector containing the TgTom22 5’ flanks. We transfected this into parasites expressing the ATc-regulated copy of TgTom22, selected parasites on chloramphenicol as described (52), and subjected parasites to negative YFP selection as described (57). We screened clones for TgTom22 knock-out using primers 21 and 22.

To generate a strain expressing the mitochondrial targeting sequence of TgHsp60 fused to c-Myc-tagged mouse DHFR, we amplified the TgHsp60 leader sequence from the vector Hsp60-RFP in pBTR (58) with BglII and AvrII, ligated this into the equivalent sites of the vector mDHFR in pBTM<sub>1</sub> (53), transfected into the TgTom22 knockdown cell line, and selected on phleomycin.

To generate a parasite strain expressing TgTom22 fused to an N-terminal DD tag, we amplified the open reading frame of TgTom22 with the primers 23 and 24, digested with Xmal and AatII, and ligated this into the equivalent sites of the vector pCTDDNm, a modified version of the vector pCTDDNh (58), which contains a c-Myc tag in place of an HA tag. We then replaced the chloramphenicol resistance marker in this vector with a phleomycin resistance marker (to generate the vector pCTDDNm-TgTom22), transfected into TgTom22 knockdown parasites, and selected on phleomycin.

To determine the correct 5’ region of the TgTom7 cDNA, we performed 5’-RACE using the SMARTer RACE cDNA amplification kit (Clontech) according to the manufacturer’s instructions. We used primer 25 for the first reaction and primer 26 for the nested reaction.

To determine the localization and function of TgTom7, we replaced the native promoter of TgTom7 with an ATc-regulated promoter, introducing an N-terminal 3xHA tag onto TgTom7 in the process. We amplified a 3’ flanks of TgTom7 with primers 27 and 28, digested the product with Xmal and NotI, and ligated into the equivalent sites of the vector pPR2-HA<sub>3</sub> (55). We next amplified the 5’ flanks of the TgTom7 gene with primers 29 and 30, digested the product with Apal and Ascl, and ligated into the equivalent sites of the pPR2-HA<sub>3</sub> vector harboring the TgTom7 3’ flank. We linearized this vector with NotI, transfected into TATi/Aku80 strain parasites (41), and selected on pyrimethamine. Clones were screened for successful promoter replacement by PCR, using primers 31 and 32, which only gives a band if the native locus is present, and primers 32 and 33, which only give a band if the 5’ region of TgTom7 is replaced by the regulated promoter.

To complement the TgTom7 knockdown mutant, we amplified the entire open reading frame of TgTom7 with primers 34 and 35 using T. gondii cDNA as template. We digested the product with NcoI and MfeI and ligated this into the equivalent sites of the pBTDDNty vector, a modified version of the pBTDDNm vector that has a 5’-Ty1 epitope tag instead of a c-Myc tag. We digested the resulting vector with AvrII and NotI, which excises the Ty1-TgTom7 open reading frame, and places the Ty1-tagged TgTom7 downstream of the constitutive β-tubulin promoter. We linearized this vector with AatII, transfected into the TgTom7 knockdown cell line, and selected parasites on chloramphenicol.

Anti-TgTom40 Antibody Generation—To generate an antibody against TgTom40, we PCR-amplified a region of the TgTom40 open reading frame (encoding residues 2–173 of the TgTom40 protein) with primers 36 and 37 and integrated the product into the vector pAVA0421 by LIC (59). The plasmid was transformed into the BL21 strain Escherichia coli, and TgTom40 expression was induced through the addition of isopropyl 1-thio-β-d-galactopyranoside. His-tagged TgTom40 was purified using nickel-nitrilotriacetic acid-agarose resin (Qiagen) according to the manufacturer’s instructions, and purified protein was used to immunize rabbits (IVMS Vet Services, Adelaide, Australia).

BN-PAGE—For preparation of proteins for BN-PAGE, parasites were filtered through a 3-μm filter and pelleted by centrifugation at 1500 x g, 10 min, 4 °C. Parasites were washed once in ice-cold phosphate-buffered saline (PBS), then solubilized to a final concentration of 2.5 x 10<sup>5</sup> parasites/μl in Native-PAGE buffer (Life Technologies, Inc.), and supplemented with EDTA-free Complete protease inhibitor mixture (Roche Applied Sciences), 2 mm EDTA, and an appropriate detergent. Samples were incubated for 30 min at 4 °C and then centrifuged at 20,000 x g for 30 min at 4 °C to remove insoluble material. Samples were separated on a 4–16% Native-PAGE BisTris gel (Life Technologies, Inc.) according to the manufacturer’s instructions, then transferred to PVDF membrane, or subjected to a second dimension SDS-polyacrylamide gel (Life Technologies, Inc.) according to the manufacturer’s instructions, before transferring to nitrocellulose membrane and subsequent Western blotting.

Sodium Carbonate Extractions, Immunoprecipitations, and <sup>35</sup>S Radiolabeling—Sodium carbonate extractions and immunoprecipitations were performed as described previously (38).

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<sup>5</sup> E. Rajendran and G. van Dooren, manuscript in preparation.
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Antibodies used for immunoprecipitations were rat anti-HA conjugated to agarose beads (Roche Applied Sciences), mouse anti-c-Myc conjugated to agarose beads (Thermo Scientific), and anti-TgTom40 antibodies conjugated to protein A-Sepharose CL-4B beads (Pierce). Co-immunoprecipitations were performed on parasite extracts solubilized in 0.5% digitonin in a buffer consisting of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, and Complete protease inhibitors.35S radiolabeling was performed as described previously (38), except that parasites were incubated in Expre35S35S protein labeling mix (PerkinElmer Life Sciences) for 10–60 min before harvesting.

Western Blotting and Immunofluorescence Assays—Western blotting and immunofluorescence assays were performed as described previously (38). Samples were probed with the following antibodies: rat anti-HA (1:100 to 1:500, Roche Applied Science); mouse anti-c-Myc (1:100 to 1:500, Thermo Scientific); mouse anti-Ty1 (1:200 to 1:1000 (60)); mouse anti-GR8 (1:100,000, a kind gift from Gary Ward, University of Vermont (61)); rabbit anti-TgTom40 (1:2000, this study); rabbit anti-

Author Contributions—G. v. D., B. S., and G. I. M. conceived the study. G. v. D. and L. M. Y. designed and performed the experiments. G. v. D., L. M. Y., B. S., and G. I. M. analyzed the data. G. v. D. wrote the manuscript with contributions from all the authors.

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