A Critical Role for *Toxoplasma gondii* Vacuolar Protein Sorting VPS9 in Secretory Organelle Biogenesis and Host Infection

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Accurate sorting of proteins to the three types of parasite-specific secretory organelles namely rhoptry, microneme and dense granule in *Toxoplasma gondii* is crucial for successful host cell invasion by this obligate intracellular parasite. Despite its tiny body architecture and limited trafficking machinery, *T. gondii* relies heavily on transport of vesicles containing proteins, lipids and important virulence-like factors that are delivered to these secretory organelles. However, our understanding on how trafficking of vesicles operates in the parasite is still limited. Here, we show that the *T. gondii* vacuolar protein sorting 9 (*TgVps9*), has guanine nucleotide exchange factor (GEF) activity towards Rab5a and is crucial for sorting of proteins destined to secretory organelles. Our results illuminate features of *TgVps9* protein as a key trafficking facilitator that regulates protein maturation, secretory organelle formation and secretion, thereby ensuring a primary role in host infection by *T. gondii*.

*Toxoplasma gondii* is an important food and waterborne pathogen causing toxoplasmosis, a usually mild disease in immunocompetent humans that can turn into a major threat in immunocompromised patients and during primary infection of pregnant woman. *T. gondii* is a member of the *Apicomplexa*, a phylum of numerous medically important parasites causing life-threatening diseases in human and animals worldwide. The phylum is typified by specific secretory organelles called rhoptries, micronemes and dense granules that are essential for host cell invasion and host pathway modulation. In *Toxoplasma*, rhoptries contain two groups, termed rhoptry (ROP) and rhoptry neck (RON), of effector proteins some of which are virulence factors; whereas micronemes secrete MIC proteins that are involved in parasite gliding, host cell attachment and invasion\(^1\). After invasion, dense granules discharge GRA proteins involved in parasitophorous vacuole (PV) formation and in hijacking host cell gene expression and metabolism\(^3\).

Despite having a single cell architecture, the parasite relies on active and abundant vesicle and protein trafficking. *T. gondii* and likely all *Apicomplexa* have reutilized classical endosomal and endocytic trafficking pathways more typical of higher eukaryotes towards building specialized secretory organelles that release parasite effectors to interplay with host cell signaling pathways as a way to take control over host immunity and ultimately to promote long-term parasitism\(^4\). It is now well established that apicomplexan parasites operate an unconventional endosome-like system (ELC) to traffic proteins from the Golgi apparatus to rhoptries and micronemes\(^5\). However, the mechanisms involved in endosome-like vesicle formation and delivery to the aforementioned organelles in general remain elusive. In mammalian cells, the endosomal system is used for the uptake of plasma membrane-associated components, which after passage through Rab5-positive early endosomes (EE) enter either Rab11A-positive recycling endosomes to return to the plasma membrane, or Rab7-positive late endosomes to be delivered to lysosomes (LE)\(^6\).

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Clearly, regulated vesicular traffic allows different cargoes to correctly reach their specific organelle destinations at the right time\textsuperscript{4–8}, and this is essential for successful parasite infection of its host\textsuperscript{17,18}. For example, dynamin-related protein B (DrpB) and clathrin, which reside in the post-Golgi network (TGN) and the endosomal-like compartment (ELC) contribute to the formation of transport vesicles that are essential for secretory organelle biogenesis\textsuperscript{11,12}. Vacular protein sorting 11 (Vps11) that is the subunit of CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and vacular protein sorting) complexes are required for transport of MIC and ROP proteins to micronemes and rhoptries of \textit{T. gondii}\textsuperscript{13}. Thus, the parasite intra-vesicular trafficking of the endolysosome pathway involves functions of the CORVET and HOPS tethering complex. In addition, \textit{T. gondii} Rab5\textsuperscript{+} and Rab7\textsuperscript{+} effector complexes likely interact with CORVET and HOPS in a manner similar to mammalian cells to induce membrane fusion within the endolysosome pathway of the parasite\textsuperscript{14,15}.

We have described that transport of MIC and ROP proteins to microneme and rhoptry organelles, respectively, also required an essential sortilin-like receptor named \textit{TgSORTLR}\textsuperscript{16} and traffic through a non-conventional ELC. The C-terminal tail of \textit{TgSORTLR} interacts with clathrin, three components of the AP1 adapter complex, Sec23/24 and three vacular protein sorting namely Vps26, Vps35 and Vps9\textsuperscript{19,20}. Furthermore, the retromer composed of Vps35-Vps29-Vps26 that recycles \textit{TgSORTLR} from TgRab5- to TgRab7-dependent ELC before delivery to Golgi, is also essential for secretory organelle biogenesis and parasite shape\textsuperscript{17}.

Here, we report that the \textit{T. gondii} counterpart of Vps9 (herein named \textit{TgVps9}) is a \textit{bona fide} Rab5 GTP-Exchange Factor (GEF) that is crucial for ROP protein maturation and processing, and its loss leads to a reduced number of rhoptries. Absence of \textit{TgVps9} also impairs peripheral microneme biogenesis and disturbs dense granule secretion resulting in an accumulation of novel vesicles present both within and outside the parasite. Together with the rrophy defect, absence of dense granule secretion severely affects parasite invasion of host cells. Collectively, these observations support the notion that \textit{TgVps9}-mediated loading of GTP to \textit{TgRab5} is crucial for fine-tuning vesicle sorting to secretory organelles, the latter being essential for \textit{T. gondii} host cell infection.

**Results**

\textit{T. gondii} vacular protein sorting 9 is a \textit{bona fide} Rab5 guanin nucleotide exchange factor. In eukaryotic cells, Vps9 domain-containing proteins are known as guanine nucleotide exchange factors (GEF) that stimulate the release of monomeric guanosine diphosphate (GDP)-bound to Rab5, allowing guanosine triphosphate (GTP) to bind and activate Rab5 that, in turn, regulates endosome vesicle trafficking\textsuperscript{16–18}. Previous work led us to identify an association with the C-terminus of \textit{TgSORTLR}\textsuperscript{16}, a protein with a predicted molecular mass of 140 kDa typified by a Vps9-like domain localized between amino acid (aa) 945 and 1117. This putative parasite Vps9 homologue harbors a region of 1326 amino acids extended at the N-terminal end and in this respect differs from its yeast and human counterparts that contain a shorter N-terminal end (Supplementary Fig. S1A). We first demonstrated that the predicted \textit{TgVps9} domain operates \textit{in vitro} as a GEF towards Rab5, by testing activity of a bacterial expressed \textit{TgVps9} recombinant protein towards human Rab5, as previously described\textsuperscript{19,20}. Based on its homology with the catalytic core of mammalian Rabex5\textsuperscript{21}; a truncated recombinant version (aa849 to aa1134) of \textit{TgVps9} was purified from \textit{E.coli}. Human recombinant Rab5A was purified in its GDP bound form and nucleotide exchange to GppNHp, a non-hydrolysable GTP analogue, was monitored by tryptophan fluorescence measurements. A dose dependent GEF activity of recombinant \textit{TgVps9} towards human recombinant Rab5A was detected and compared to human recombinant Rabex5 (Fig. 1A). As expected, no tryptophan fluorescence change was observed in the presence of excess GDP as no conformational change was induced during nucleotide exchange from GDP to GDP (Fig. 1A).

\textit{TgVps9} localizes to the endosome-like compartment of \textit{T. gondii}. Having established that recombinant \textit{TgVps9} possesses GEF activity towards recombinant Rab5, we then sought to determine in \textit{T. gondii} whether the protein resides in the same subcellular compartment as Rab5. To this end, we chromosomally appended the hemagglutinin (HA) epitope at the 3’-end of the endogenous \textit{TgVps9} gene and validated by western blot that the tagged \textit{TgVps9} was readily expressed by transgenic tachyzoites (Supplementary Fig. S1B). HA-tagged \textit{TgVps9} migrated with an apparent molecular mass of 170 kDa, which is higher than the predicted 140.0 kDa, likely due to the observation that \textit{TgVps9} is heavily phosphorylated with 30 different phospho-sites indicated in Supplementary Figure S1A and collated at ToxoDB (www.toxodb.org).

Consistent with its \textit{in vitro} GEF activity towards Rab5\textsuperscript{19,20}, \textit{TgVps9}-HA \textit{in vivo} co-localized with TgRab5A (Fig. 1B, top and left panel). As expected, we confirmed a co-localization between \textit{TgVps9} and \textit{TgSORTLR} (Fig. 1B, top panel), the endosomal-like compartment (ELC) receptor that has been used to efficiently pull down \textit{TgVps9}\textsuperscript{10}. The unprocessed precursor pro-RP4 (Fig. 1B, top panel) known to be present in the ELC also co-distributes with \textit{TgVps9}, while surprisingly proM2AP, a microneme marker, does not (bottom panel). In addition, VP1 a marker of the plant-like vacuole that is present in close vicinity to ELC co-distributes with \textit{TgVps9} (Fig. 1B, top panel). In contrast, CPL (lysosomal-related compartment marker), GRASP (Golgi reassembly stacking protein), M2AP (MIC2-associated protein) or ROP4 (rhoptry marker) do not co-distribute with \textit{TgVps9} (bottom panel). Taken together, these co-localization studies indicate that \textit{TgVps9} is embedded in the endosomal-like compartment (ELC) together with Rab5, a compartment with an established role in the formation of secretory organelles of \textit{T. gondii}\textsuperscript{4–8,10}.

Conditional ablation of \textit{TgVps9} affects secretory organelle biogenesis to generate large novel vesicular-like structures. To examine \textit{TgVps9} function in a clonal homogenous parasite population, we generated conditional anhydrortedacyclin (ATC)-inducible knockout \textit{TgVps9} mutants (named \textit{iKOTgVps9}) using the strategy depicted in Fig. 2A. We selected several positive clones from the emerging stable parasite population
and the genome editing of one expanded clone in vitro was verified by PCR using the two specific primers (see nucleotide sequences in Methods) shown in blue arrow (Fig. 2A), thus demonstrating the perfect integration of the knock-out vector at the TgVps9 locus (Fig. 2B). Following ATc treatment, while a significant reduction of HA-TgVps35 protein was observed 24 h post-treatment, 48 h or 72 h of ATc-treatments were required for a complete and reproducible disappearance of HA-TgVps35 protein by western blotting (Fig. 2C). We confirmed these latter observations by confocal imaging (Fig. 2D) and further investigated all phenotypic consequences of this ATc-inducible TgVps9 knock out mutant at least at 48 h post-treatment. Next, we examined iKO TgVps9 mutants by electron microscopy and observed several striking ultrastructural changes associated with the loss of TgVps9. Both apical (yellow arrows) and peripheral micronemes (white arrows) were observed in cytoplasm anterior to...
the nucleus of ATc-untreated tachyzoites (Fig. 3A,B) whereas ATc-treated iKO TgVps9 parasites, micronemes were only observed in the apical tip close to the conoid (Fig. 3C–E). We counted a total number of 30 micronemes located at the extreme apical end of 43 ATc-treated iKO- TgVps9 mutants using electron microscopy while a total number of 199 apical and peripheral micronemes were seen in 29 ATc-untreated parasites, indicating that there were about 5-fold fewer micronemes in TgVps9-deficient mutants versus the parental strain. Clearly, these data indicate the absence of peripheral micronemes in ATc-induced iKO TgVps9 mutants and the presence in the cytoplasm at the proximity of the nucleus of a novel large vesicular structure of approximately 500-nm diameter (panel D, black arrows). Elevated numbers of novel vesicles of variable size and morphology were also observed in the PV space delimited by the PVM (panel E, shown with *). We estimated that about 17% of iKO TgVps9 mutants examined by electron microscopy contained these aforementioned 500-nm intra-parasite vesicles while approximately 20% of mutants had novel vesicles of variable size and morphology in their PV space. In addition, we observed a significant reduction of the overall number of rhoptries per mutants using electron microscopy. Specifically, ATc-treated iKO TgVps9 mutants contained less than two thirds the relative number of rhoptries per mutant compared to the parental line. Also, we observed a disorganized ultrastructural morphology with the marked absence of the typical banana-shaped bodies in several TgVps9-depleted mutants (Fig. 3D), in a manner similar to the retromer iKO TgVsp35 mutants’ whereas untreated iKO TgVps9 parasites appeared structurally normal with all secretory organelles (Fig. 3A,B). These latter observations suggest that the cytoskeleton of parasite bodies may also be affected in these mutants. It should be mentioned that electron microscopy was used to show that other organelles including the mitochondrion, the nucleus, the Golgi apparatus, the inner complex membrane (IMC) and the plasma membrane appeared morphologically normal in these iKO TgVps9 mutants treated with ATc for 48h (Supplementary Fig. S2, see panel A–E). In addition, iKO TgVps9-deficient mutants appear to undergo normal endodyogeny with two daughters forming within the mother cell (Supplementary Fig. S2F, see stars indicating the nucleus of two dividing daughter tachyzoites). Collectively, these data suggest that the traffic to and the integrity of the other parasite organelles were not altered by the loss of TgVps9. Furthermore, rhoptries, micronemes and dense granules were not completely absent either or not morphologically affected per
se in TgVps9-deficient mutants (Fig. 3 and Supplementary Fig. S2), only organelle number was reduced in these mutants. Taken together, these data indicate that TgVps9 likely regulates the turnover of vesicle precursors and pre-organelles destined to become fully mature secretory organelles.

The loss of TgVps9 causes aberrant organelle secretion. We also examined the phenotypic consequences of TgVps9 loss on the subcellular localizations of different secretory organelle markers. In ATc-induced iKOTgVps9 mutants, ROP2-3 and ROP4 proteins were abnormally sorted into the host cytoplasm and decorated the host cell nuclear envelope (Fig. 4A,B, right panels, see white arrows). In the absence of ATc, iKOTgVps9 mutants displayed normal apical localization of ROP proteins (Fig. 4A,B, left panels). In ATc-induced iKOTgVps9 parasites, pro-ROP4 was also profoundly mis-sorted (Fig. 4C, right panels) with diffuse and weak labeling in both the PV space and the host cell cytoplasm (white arrow) unlike non-ATc-induced parasites that showed the typical apical pre-rhoptry localization of pro-ROP4, i.e. proximal to parasite nuclei (Fig. 4C, left panels). The imaging data suggest that the iKOTgVps9 mutant-hosting PV may also be leaky following ATc induction for 48 h, thus resulting in the diffusion of pro-protein and mature protein in the vacuolar space and beyond the PV. Nevertheless, these data indicate that TgVps9 loss results in an accumulation of ROP precursor proteins, their
Figure 4. Conditional ablation of TgVps9 results in mis-sorting of ROP proteins. (A) Confocal immunofluorescences of ROP2-3 proteins in iKOTgVps9 mutants in the presence (right panels) or absence of ATc (left panels) using specific antibodies to ROP2-3. (B) Confocal immunofluorescences of ROP4 proteins in iKOTgVps9 mutants in the presence (right panels) or absence of ATc (left panels) using specific antibodies to ROP4. (C) Confocal immunofluorescences of proROP4 proteins in iKOTgVps9 mutants in the presence (right panels) or absence of ATc (left panels) using specific antibodies to proROP4. Upper panels in (A–C) images correspond to small vacuoles containing 16 or less daughter parasites. Lower panels represent large vacuoles containing 32 or more daughter parasites. Scale bar on all images correspond to 10 μm.
mis-sorting to other subcellular compartments and a significant reduction of the number of rhoptries per parasites as observed by electron microscopy.

We confirmed in the conditional TgVps9 mutants that the typical conical microneme M2AP and MIC3 signals (Fig. 5A, left panels, red arrows) were completely changed to fluorescence signals at the extreme tip of each TgVps9-depleted mutant (Fig. 5A, right panels, yellow arrows). The most impressive and marked mis-sorting affects the dense granule GRA3 that was exclusively retained within the PV space (Fig. 5B, right panels) in the iKOTgVps9 mutants whereas this protein decorated the PV membrane of vacuoles containing parental parasites, as expected (Fig. 5B, left panels, white arrows). The location of TgSORTLR was unchanged (Fig. 5C), confirming that not all proteins in the secretory ER–Golgi and ELC pathways are mis-sorted in TgVps9-deficient parasites. Altogether, these data clearly indicate that TgVps9 is required for correct protein trafficking, sorting and delivery to the three main secretory organelles: rhoptry, microneme and dense granules.

**Conditional TgVps9 silencing dysregulates ROP protein maturation.** In T. gondii, formation of rhoptries and micronemes is correlated with proteolytic processing followed by maturation of ROP and MIC proteins, respectively. Therefore, we investigated the role of TgVps9 in the processing and maturation of representative ROP and MIC proteins. Specific antibodies that exclusively recognized the N-terminal pro-peptides of ROP4 revealed enhanced accumulation of pro-protein ROP4 in TgVps9-deficient mutants (Fig. 6A, left panel, single star). By calculating the ratio of the pro-protein to the mature ROP4 protein, we estimated that pro-ROP4 protein level was increased to almost 2-fold in ATc-treated iKOTgVps9 mutants compared to untreated parental parasites (Fig. 6F). Using specific antibodies to the mature ROP4 protein, we detected a rise of proROP4 protein level to 4-fold (Fig. 6B, single stars), suggesting that pro-ROP4 protein accumulated between 2- to 4-fold higher in TgVps9-deficient mutants versus parental parasites (compare Fig. 6B,F, single star). As a consequence, the amount of processed mature ROP4 diminished (Fig. 6B,F, right lane, double stars). Similarly, we observed an increase level of unprocessed proROP2 (Fig. 6C,F, single star) that was estimated to be approximately 3-fold with a reduced amount of the mature ROP2 protein. We observed no significant changes for pro-M2AP protein (Fig. 6D–F, single star) or processed M2AP protein (Fig. 6E,F, double stars). We therefore concluded that pro-ROP proteins specifically accumulated in iKOTgVps9 mutants, suggesting that TgVps9 is likely involved in the delivery of both proteases and pro-ROP proteins to the same subcellular compartment and that their proteolytic processing is important for rhoptry organelle formation.

**Conditional TgVps9 silencing abrogates host cell invasion by T. gondii.** More importantly, homogenous clonal populations of iKOTgVps9 mutants allowed us to address the role of TgVps9 in T. gondii infection. The iKOTgVps9 mutants were severely impaired in their ability to invade host cells with an 80% decrease after 48 h of ATc-treatment (Fig. 7A). In addition, at 7 day post-infection, TgVps9-depleted mutants did not form plaques associated with multiple rounds of host cell invasion in the presence of ATc (Fig. 7B), indicating that TgVps9 is essential for ensuring proper formation of secretory organelles that are necessary for parasite propagation through multiple cycles of invasion, lysis and reinvasion of host cells. We have not observed any obvious deficiency in parasite egress from the host cell as the ATc-treated iKOTgVps9 mutants spontaneously lysed out and freshly egressed tachyzoites can be recovered out at 72 hours post-infection. Thus, we suggest that the reduction in number of secretory organelles per parasite and their default in organelle secretion represent the critical functions of TgVps9 that are required for proper host infection by T. gondii.
Figure 5. Conditional TgVps9 silencing affected microneme biogenesis and dense granule secretion. (A) Confocal immunofluorescences of M2AP and MIC3 proteins in iKO TgVps9 mutants in the presence (right panels) or absence of ATc (left panels) using antibodies specific to each protein, respectively. Note that the typical and conical signal of M2AP and MIC3 proteins (red arrows) in the parental strain disappears. Instead, only residual punctuated signal was seen in TgVps9-depleted mutants (yellow arrows), indicating the absence of peripheral micronemes in these mutants. The whole bodies of intracellular parasites were shown by phase contrast in order to indicate fluorescence signals corresponding to micronemes located at the extreme apical end of Cas9-GFP positive parasites (yellow arrows). (B) Confocal immunofluorescences of GRA3 protein in iKO TgVps9 mutants in the presence (right panels) or absence of ATc (left panels) using specific antibodies to GRA3. Note the absence of GRA3 protein delivery to the parasitophorous vacuole membrane (PVM) that contrasts to the situation in the parental parasites (white arrows). (C) The presence of TgSORTLR in the Golgi-ELC region was unchanged in parental parasites and mutants regardless of treatment with ATc or not. Scale bar is 10 μm.
As discussed above, TgVps9 displays *in vitro* GEF towards human Rab5A, comparable to that of Rabex5. It follows then that some phenotypic traits of TgVps9-deficient mutants resemble those previously reported for TgRab5A protein after its encoding gene has been disrupted. As both TgRab5A and Vps11 probably interact through the CORVET-tethering complexes, this could explain their phenotypic similarities with those observed for iKOTgVps9 mutants described here.

Protein trafficking that relies on VPS9 has been described in several other eukaryotic cells such as yeast, which has three Vps9-domain containing proteins, Vps9, Muk1 and Vrl1, all exhibiting GEF activity towards Rab5 paralogs. Mammalian cells contain at least nine Vps9 domain-containing proteins fulfilling diverse functions including regulation of protein transport, endocytosis and signaling pathways. Additionally, it has been reported that Vps9 domains also interact with retromer complex and phosphatidylinositol 3-phosphate (PI3P) to promote the enrichment of PI3P lipids at the endosomes. Knowing that TgSORTLR and the retromer machinery in T. gondii share similarities with those of TgVps9, the latter may participate in regulation of retromer and endosomal lipid content. However, TgVps9 is not associated with the Golgi apparatus like TgSORTLR, suggesting that this parasite Rab5 GEF is likely involved in anterograde transport and secretory organelle formation, rather than protein recycling in T. gondii.

**Methods**

**Parasite culture.** We used T. gondii tachyzoites of RH strain for CRISPR/Cas9 knockout experiment, RHΔKu80 for the knock in of TgVps9 gene (TGME49_230140) and RHΔKu80TATi for inducible knockout (iKO) strain that were grown using Human Foreskin Fibroblast (HFF) cells from ATCC (USA) as described. The iKOTgVps9 mutants were cultured in the presence of 1.5 μg/ml anhydrotetracycline (ATc).
Production of recombinant TgVps9, Rabex5, and Rab5 proteins. Recombinant protein of the catalytic core of TgVps9 from aa849 to aa1134 was generated using a modified pET19 plasmid that expresses His-tag protein with a TEV cleavage site using the following primers: forward (Recomb-Vps9a.d4_F) CCGGGATCCATATGGCGTCTTCTGCCTCTTTTTCTGCC and reverse (Recomb-Vps9a.d4_R) CCGGGATCTTAGCGTTCGCGTTCGCGGTCGTATTC. Human recombinant Rabex5 from aa132 to aa397 and full length Rab5a with a C-terminal CVIL mutation were prepared as previously described. The recombinant TgVps9 was expressed in BL21 Codon Plus (DE3)-RIPL, and cell pellet was resuspended in buffer A (50 mM HEPES pH 8, 300 mM NaCl, 1 mM Tris (2-carboxyethyl) phosphine (TCEP), 20 mM imidazole pH 8), sonicated, and centrifuged...
at 20,000 rpm for 1 hour at 4 °C. Cell lysate was incubated with 2 ml agarose Ni-NTA beads, washed with buffer A and subsequently buffer B (50 mM HEPES pH 8, 300 mM NaCl, 1 mM TCEP, 30 mM imidazole pH 8). Bound protein was eluted using buffer C (50 mM HEPES pH 8, 300 mM NaCl, 1 mM TCEP, 500 mM imidazole pH 8). His-tags were removed by TEV protease cleavage during overnight dialysis in Buffer A without TCEP and imidazole. In order to remove the His-tagged TEV protease, dialyzed protein solution was incubated with Ni-NTA beads again before the GEF assays were performed.

GEF assay. GEF activities were analyzed by intrinsic tryptophan fluorescence measurements showing fluorescence changes due to the conformational change from GDP to GTP state19,20. Rab5 fluorescence was excited at 297 nm and emission signals were detected at 340 nm. The fluorescence was recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). 10 μM of 5′-Guaneryl imidodiphosphate (GppNHp), non-hydrolysable analog of GTP, or 10 μM GDP were added to 1 μM of Rab5 GDP in GEF assay buffer (25 mM HEPES pH 7.5, 200 mM NaCl, 1 mM MgCl2, 2 mM diithioerythritol (DTE)), subsequently 200 nM or 400 nM of TgVps9 or 200 nM of Rabex5 were added.

Generation of stable transgenic strains. Endogenous gene tagging methodology using pLIC-HA-DHFR plasmid24 was used to generate TgVps9-HA knock in parasites. DNA of TgVps9 was cloned in this plasmid using the following primers: forward (F-Kl_Vps9) TACTTCCAATCCAATTTAATGCCCCTGGTGTCCGTCC and the reverse (R-Kl_Vps9) TCCCTCCATTCATTAGCTTCTCAGCTAATG. To obtain iKO-TgVps9 mutants, we used pG13-D-T7S4 plasmid25 in which a 2-kb DNA containing the promoter sequence was cloned using the following primers: forward (iKO-TgVps9-5′_F) CCGGCCATATGCTTCTAACGGCACCACTTAAGGTGC and reverse (iKO-TgVps9-5′_R) CCGGCTAATGCTTCTAAGGGCACCACTTAAAGGTGC and another 2-kb DNA containing the coding sequence of TgVps9 gene using the following primers: forward (iKO-TgVps9-3′_F) CCGGCTGATCATATGCTTCTACATGATTTAAGGTGC and reverse (iKO-TgVps9-3′_R) CCGGCTTACGAGGGGAGAAGGAGAAGACACATCTCAGCTACGACC with the HA-tag sequence underlined in the forward primers inserted at the N-terminus of TgVps9 protein, right after the initiation ATG codon. 1 × 10^6 parasites were transfected with 50 μg of linearized plasmid and selected with 2 μM of pyrithamine. The emerging pyrimethamine-resistant population was selected by cloning dilution. The clones were checked for plasmid integration by PCR using genomic DNA and two primers: forward (named A or Test_iKOVP9.5_F) ATTACAGCCACGAGTGGCCACCGAAT and reverse (named B, DHFR-int. R) GCGTCTAGCTTCTGCGAGAGGGAGAAGGAGAAGACACATCTCAGCTACGACC.

Immunofluorescence microscopy. Confocal microscopy was performed as described previously30,38. Briefly, intracellular parasites within HFF cells on 24-well coverslips were fixed by 4% paraformaldehyde for 10–15 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 and blocked with 5% fetal bovine serum (FBS). These cells were incubated with primary antibodies for 1 h at 37 °C and sequentially stained with secondary antibodies conjugated with Alexa 488, 594 or 647 in addition to DAPI for 30–45 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 and blocked with 5% FBS, incubated with primary antibodies for 1 h at 37 °C and sequentially stained with secondary antibodies conjugated with Alexa 488, 594 or 647 in addition to DAPI for 30–45 min at 37 °C. Stained cells were mounted with Mowiol. All images were captured by a confocal microscope LSM780 or 880 (Carl Zeiss). Image processing was performed by open-source Fiji software.

Western blots. Intracellular iKO-TgVps9 mutants or parental parasites incubated with ATc or not for 48 h were scrapped and washed twice with PBS. The intracellular parasites were pelleted before suspension by Laemmli buffer (62.5 mM Tris-HCl pH 6.8; 2% SDS; 100 mM DTT; 10% sucrose) and boiled for SDS-PAGE. 2 × 10^7 parasites were fractionated on 10% acrylamide gels, which were transferred to nitrocellulose membranes as previously described39. Immunoblot was performed using several anti-MIC and ROP antibodies in TNT buffer (100 mM Tris-HCl pH 7.6; 150 mM NaCl; 0.1% Tween20). All membranes were stained with antibodies specific to the glycolytic enzyme anti-ENO240 as a loading control after stripping antibody.

Electron microscopy. For transmission electron microscopy, cells were fixed in 2.5% glutaraldehyde for 10–15 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 and blocked with 5% fetal bovine serum (FBS). These cells were incubated with primary antibodies for 1 h at 37 °C and sequentially stained with secondary antibodies conjugated with Alexa 488, 594 or 647 in addition to DAPI for 30–45 min at 37 °C. Stained cells were mounted with Mowiol. All images were captured by a confocal microscope LSM780 or 880 (Carl Zeiss). Image processing was performed by open-source Fiji software.

Invasion and plaque assays. Wild type RHΔKu80TATi strain and iKO-TgVps9 parasites were incubated under ATc condition for 48 h and mechanically lysed by passage through a syringe. 1 × 10^6 parasites were inoculated to HFF cells and incubated for 1 h at 37 °C. After infection for 1 hour, extracellular parasites were washed out with PBS and used to infect HFF cells before growing for 24 h at 37 °C. These infected cells were fixed by PFA and sequentially stained by GAP45 antibody with DAPI and counted by Axioimager Z1 (Carl Zeiss). Host cell invasion values were calculated using the ratio of intracellular parasite/host nucleus numbers as described41. For plaque assays, 400 freshly lysed parasites were used to infect HFF cells followed by incubation for 7 days with or without ATc. These cells were fixed by ethanol and stained by crystal violet.

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Acknowledgements

We would like to thank Prof. David J. P. Ferguson (Oxford University, UK) for critically reading this manuscript, Dr Roger S. Goody (MPI for molecular Physiology, Dortmund) for providing plasmid DNA for the expression of Rab5 and Rabex5; and Dr Tchilabalo Dializioto Alayi for quantification of western blots. This work was supported by the following grants: Laboratoire d’Excellence (LabEx) ParaFrap from the National Agency for Research ANR-
11-LABX-0024 and the ANR-14-CE14-0002-01. We also acknowledge additional financial support from the INSERM, Pasteur Institute of Lille, CNRS and the DFG/ANR grant GO 284/8-1. Post-doc and PhD fellowships were from LabEx ParaFrap to T.S. and F.S., respectively. M.A.H is supported by the European Research Council (ERC Consolidator grant no. 614880).

**Author Contributions**

T.S. conceived and designed the experiments, analyzed data and wrote paper; F.S. and M.A.H. involved in reverse genetics approaches and wrote paper; C.S., electron microscopy; L.K.O. and H.B., involved in testing GEF activity; G.L. involved in GEF activity design; S.T. designed, supervised and wrote this study. The manuscript has been seen and approved by all authors.

**Additional Information**

Supplementary information accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Sakura, T. *et al.* A Critical Role for *Toxoplasma gondii* Vacuolar Protein Sorting VPS9 in Secretory Organelle Biogenesis and Host Infection. *Sci. Rep.* **6**, 38842; doi: 10.1038/srep38842 (2016).

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