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**The merozoite-specific protein, TgGRA11B, identified as a component of the  
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1 *Succinctus*

2 **The merozoite-specific protein, TgGRA11B, identified as a component of the *Toxoplasma gondii***  
3 **parasitophorous vacuole in a tachyzoite expression model**

4 Chandra Ramakrishnan<sup>a#\*</sup>, Robert A. Walker<sup>a#</sup>, Ramon M. Eichenberger<sup>a</sup>, Adrian B. Hehl<sup>a</sup>, Nicholas  
5 C. Smith<sup>b</sup>

6 <sup>a</sup> Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, CH-8057, Zurich,  
7 Switzerland and <sup>b</sup> Research School of Biology, Australian National University, Canberra, ACT, 2601,  
8 Australia.

9 # Equal contributions

10 \* Correspondence: Tel: +41 786 760 543, email: [chandra.ramakrishnan@uzh.ch](mailto:chandra.ramakrishnan@uzh.ch)

11

12 **Abstract**

13 The apicomplexan, *Toxoplasma gondi*, infects all warm-blooded animals as intermediate hosts but only  
14 felids as definitive hosts. Dense granule proteins are critical for the survival of *Toxoplasma* within host  
15 cells but, whilst these proteins have been studied intensively in tachyzoites, little is known about their  
16 expression in the coccidian stages in the cat intestine. Transcriptome profiling indicates that two  
17 putative dense granule proteins, TgGRA11A and TgGRA11B, are expressed uniquely in merozoites.  
18 Immunofluorescent microscopy of *Toxoplasma*-infected cat intestine and tachyzoites engineered to  
19 express TgGRA11B, reveals that it is a dense granule protein that traffics into the parasitophorous  
20 vacuole and its membrane.

21

22 **Keywords**

23 *Toxoplasma gondii*, Coccidia, merozoite, TgGRA11B, dense granule, parasitophorous vacuole

24 *Toxoplasma gondii* is an apicomplexan parasite with a unique ability to infect, seemingly, any warm-  
25 blooded animal as an intermediate host but with felids as the only definitive host. It consequently has a  
26 very complex life cycle. In keeping with this complexity, *T. gondii* possesses four invasive forms –  
27 sporozoites, bradyzoites, tachyzoites and merozoites – the former two being involved in transmission  
28 from host to host and the latter two being critical for rapid population expansion by asexual  
29 reproduction in the intermediate and definitive hosts, respectively (Ferguson, 2004). All four of these  
30 forms of the parasite have the ability to attach to, invade and modulate host cells, mobilising proteins  
31 on their surface and within intracellular organelles such as micronemes, rhoptries and dense granules in  
32 an exquisitely well-coordinated sequence that results in the formation of a membrane-bound  
33 parasitophorous vacuole around the parasites, within the cytoplasm of the host cell (see Carruthers and  
34 Boothroyd, 2007; Mercier and Cesbron-Delauw, 2015 for reviews).

35 Dense granule proteins (GRAs) are pivotal to the maturation and maintenance of the parasitophorous  
36 vacuole. Some GRAs are involved in the formation of an intravacuolar network, others in mediating  
37 uptake of nutrients from the host and a third group is exported across the parasitophorous vacuole  
38 membrane into the host cytoplasm and nucleus, modulating the host cell cycle and immune response  
39 (reviewed by Mercier and Cesbron-Delauw, 2015). Thus far, forty GRAs have been identified in *T.*  
40 *gondii*, mostly in tachyzoites (Nadipuram et al., 2016), though many are also found in sporozoites and  
41 bradyzoites (Fritz et al., 2012a, 2012b; Behnke et al., 2014). Although dense granules remain a  
42 conserved feature in merozoites, the invasive and rapidly replicating stages of the coccidian life cycle  
43 in the feline intestine, there is very little overlap between the expression profiles of individual GRAs in  
44 this stage compared with the other rapidly replicating form of *T. gondii*, the tachyzoite (Behnke et al.,  
45 2014; Hehl et al., 2015); indeed, until recently, only two GRAs have been found to be definitely  
46 expressed in merozoites (NTPase 1 and GRA7) and both of these are expressed across different stages  
47 of the life cycle (Ferguson et al., 1999a, 1999b; Ferguson, 2004). However, our transcriptome profiling

48 revealed that the *gra* genes with the highest transcript levels in merozoites of *T. gondii*, *gra11a*  
49 (TGME49\_212410) and *gra11b* (TGME49\_237800), are significantly downregulated in the tachyzoite  
50 stage (Hehl et al., 2015); the definitive localisation of these unique GRAs has not yet been addressed.

51 TgGRA11A and TgGRA11B share 66% identity at the amino-acid level, with both predicted to  
52 comprise a signal peptide and three transmembrane domains (Fig. 1a - TgGRA11B). However, our  
53 homology searching failed to uncover significant homology to any other previously characterised  
54 protein or protein domain. Therefore, in order to investigate a potential role for TgGRA11B in  
55 merozoite biology, localisation studies were carried out using polyclonal mouse sera raised against a  
56 recombinant protein that corresponds to the more divergent region of TgGRA11B (Fig. 1a). The  
57 recombinant TgGRA11B protein was expressed in bacteria using the pET41a(+) system (Novagen®)  
58 and purified under denaturing conditions using NiNTA agarose (QIAGEN) as per manufacturer's  
59 instructions, and polyclonal mouse sera was produced subsequently, as described previously (Walker et  
60 al., 2015). Immunofluorescent microscopy, using this antisera, showed staining consistent with the  
61 localisation of TgGRA11B within dense granules of intracellular merozoites in feline enterocytes (Fig.  
62 1 b-e) at early (days 3 and 5 p.i.) stages of asexual development. Later in infection (day 7 p.i.), staining  
63 of infected enterocytes in vivo indicates that TgGRA11B traffics, first, to the parasitophorous vacuole  
64 and, subsequently, to the parasitophorous vacuole membrane (Fig. 1 f-g). Unfortunately, however,  
65 definitive assignment of localisation of TgGRA11B to merozoite dense granules and parasitophorous  
66 vacuoles in vivo is currently not possible because of a lack of verified specific markers for these  
67 structures in the coccidian stages of development of *T. gondii*. Therefore, we engineered a tachyzoite  
68 expression model for TgGRA11B (Fig. 2).

69 Construction of the pTubA-TgGRA11B-HA3-mCHERRY-dhfr:Lox-dhfr-DHFR-sag1-Lox (Fig. 2a)  
70 plasmid was carried out as follows: first, the 711 bp coding sequence of mCherry and adjacent 3'  
71 untranslated region (UTR) of dihydrofolate reductase-thymidylate synthase (*dhfr*) was PCR amplified

72 from the pmCherry.LIC.DHFR plasmid (Huynh and Carruthers, 2009) using primers  
73 **ATGCATATGGTGAGCAAGGGCGAG** (*nsii*) and TGTCACTGTAGCCTGCCAGA and cloned into  
74 pGEM®-T Easy (Promega) via AT-cloning; second, plasmid sequencing was carried out to select  
75 clones where the pGEM®-T Easy plasmid *nsii* site was positioned 62 bp from the insert *nsii* site; next,  
76 the minimal 493 bp alpha tubulin (*tuba* or *tgme49\_316400*) promoter and adjacent start codon were  
77 amplified from CZ *T. gondii* genomic DNA using primers  
78 **ATGCATGGATCCATCGTCTAGAGTCCC**CGTTCGTGAA (*nsii*) and  
79 **ATGCATTTTGT**CGAAAAAGG (*nsii*) and cloned into pmCHERRY-dhfr via *nsii*; third, the 84 bp  
80 coding sequence for a triple haemagglutinin tag (HA3) was PCR amplified from pHA3.LIC.DHFR  
81 (Huynh and Carruthers, 2009) using primers **CATATGGATATCTACCCGTACGACGTCCCG** (*ndei*,  
82 *ecory*) and **CATATGGGCATAATCTGGAACATCGTAAG** (*ndei*) and cloned into pTubA-  
83 mCHERRY-dhfr via *ndei*; fourth, the entire 1,413 bp coding sequence of *tggra11b* (*TGME49\_237800*),  
84 excluding the stop codon, was PCR amplified from CZ *T. gondii* genomic DNA using primers  
85 **ATGTCCCACCGCATGGCAT** and **TGGCTTCAACTCGTCCTCTTCC** and inserted in-frame with  
86 the HA3-mCHERRY coding sequence by blunt-end cloning; a Lox-flanked dhfr-DHFR-sag1 plasmid  
87 was created in parallel by PCR amplifying the pyrimethamine-resistant DHFR with its own promoter  
88 from pHA3.LIC.DHFR (Huynh and Carruthers, 2009) using primers **ATCGATAAGCTTCGCCAGG**  
89 (*clai*) and **TTAATTAAACAGCCATCTCCATCTGGAT** (*paci*) and cloning into pLox-TubA-CAT-  
90 sag1-Lox (Brecht et al., 1999) via *clai* and *paci* (i.e. replacing the TubA-CAT sequence); and, finally,  
91 the Lox-flanked pyrimethamine-resistant *dhfr* cassette was inserted adjacent to the TubA-TgGRA11B-  
92 HA3-mCHERRY-dhfr cassette via *noti* and *apai*.

93 The entire pTubA-TgGRA11B-HA3-mCHERRY-dhfr:Lox-dhfr-DHFR-sag1-Lox reporter cassette was  
94 PCR amplified using vector-specific M13R and M13F primers and co-transfected as donor DNA into  
95 CZ *T. gondii* tachyzoites with the pCas9:sgUPRT plasmid and selected with 1 µM pyrimethamine, as

96 described previously (Shen et al., 2014). CRISPR/Cas9 mediated integration of the construct at the  
97 *tguprt* genomic locus (exon 5) was confirmed for individual pyrimethamine-resistant clones by PCR  
98 screening using primers GCATGCAGCCATATACGAAA and GCCTATCGCTCTTCACCTTCT  
99 (PCR 1; 600 bp) and GCATGCAGCCATATACGAAA and ATGCATTTTGTGCGAAAAAGG (PCR 2;  
100 962 bp) (Fig. 2a). PCR analysis of the pyrimethamine-resistant clone 1 revealed the correct insertion of  
101 the reporter cassette into exon 5 of *tguprt* (Fig. 2b).

102 Antibodies to both HA and recTgGRA11B co-localise with putative dense granules (Fig. 2c) and the  
103 parasitophorous vacuole (Fig. 2d) of tachyzoites engineered to express TgGRA11B, when using  
104 permeabilisation with either 0.25% Triton X-100 or 0.01% saponin, respectively. Antibodies to HA  
105 also co-localise with the antibody to the bona fide dense granule protein after permeabilisation with  
106 Triton X-100, GRA1 (Fig. 2e). Furthermore, the same two antibodies label the parasitophorous vacuole  
107 of TgGRA11B-expressing tachyzoites under gentle permeabilisation conditions with saponin (Fig. 2f).  
108 Counterstaining with the tachyzoite surface protein, SAG1, clearly distinguishes between the parasite  
109 plasma membrane and the parasitophorous vacuole, which is labeled with anti-HA antibodies (Fig. 2g).  
110 Finally, immunofluorescent microscopy detected mCherry signal in putative dense granules and the  
111 maturing parasitophorous vacuole of tachyzoites expressing the merozoite-specific TgGRA11B-(Fig.  
112 2h).

113 In conclusion, using immunofluorescent microscopy on sections of *Toxoplasma*-infected cat intestine  
114 and by using a novel model where tachyzoites are engineered to express the merozoite-specific  
115 TgGRA11B, we have been able to demonstrate that this protein is, indeed, a dense granule protein.  
116 Furthermore, we present evidence that it is trafficked from parasites into the maturing parasitophorous  
117 vacuole and, subsequently, into the parasitophorous vacuole membrane surrounding *T. gondii* within its  
118 host cell – in this case, uniquely, the enterocyte of the feline intestine. This is in keeping with the  
119 protein's possession of an N-terminal hydrophobic signal peptide and three hydrophobic  $\alpha$ -helices,

120 features that, in numerous other GRAs, are associated with secretion into the parasitophorous vacuole  
121 and subsequent localisation in its membrane (Mercier and Cesbron-Delauw, 2015).

## 122 **Ethics statement**

123 Experiments involving animals were performed under the direct supervision of a veterinary specialist,  
124 and according to Swiss law and guidelines on Animal Welfare and the specific regulations of the  
125 Canton of Zurich. Permit number 108/2010 covers all animal experiments presented in this paper and  
126 was approved by the Veterinary Office and the Ethics Committee of the Canton of Zurich (Kantonales  
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179

180 **Figure 1. In vivo localisation of native TgGRA11B in a *Toxoplasma gondii*-infected cat intestine.**

181 (a) Schematic representation of the predicted protein structure of TgGRA11B including the location of

182 a signal peptide (SP) and three transmembrane domains (TM1, TM2, TM3). Polyclonal antisera were

183 raised against recTgGRA11B, a bacterially expressed recombinant protein corresponding to a region of

184 the predicted TgGRA11B protein structure that encompasses TM2 and TM3 (indicated by a horizontal

185 black line). (b-g) Sections of paraffin-embedded *T. gondii*-infected cat intestine from different post-

186 infection time-points with the CZ strain (Hehl et al., 2015) were assessed by immunofluorescent

187 microscopy. At early stages of *T. gondii* asexual expansion at days 3 (b, c) and 5 (d) post-infection with

188 tissue cysts of CZ *T. gondii* (as described by Hehl et al., 2015), merozoites (surface-stained in green

189 with polyclonal antisera from a sheep naturally infected with *T. gondii*; Hehl et al., 2015) are

190 characterised by TgGRA11B localisation (red) that is consistent with dense granule cargo. In later

191 stages of merozoite development, at day 7 post-infection, TgGRA11B (green) is detected not only in

192 these putative dense granules (e, f) but also in the parasitophorous vacuole and parasitophorous vacuole

193 membrane (f, g). DAPI counterstain was used for nuclear staining (b-g). The antisera to recTgGRA11B

194 did not label any other stages of development of *T. gondii*. Scale bars are 5µm.

195

196 **Figure 2. Genetic engineering and immunolocalisation of TgGRA11B in a tachyzoite expression**  
197 **system.** (a) Schematic representation of the *tubA-TgGRA11B-HA3-mCHERRY-dhfr:Lox-dhfr-DHFR-*  
198 *sag1-Lox* reporter construct used for site specific integration into the *TgUPRT* genomic locus in wild-  
199 type CZ *T. gondii* tachyzoites (WT). A clonally isolated mutant (clone 1) is also schematically  
200 represented, demonstrating site-specific insertion of the reporter construct. (b) Diagnostic PCR  
201 demonstrated correct insertion of the reporter construct in clone 1 (with a positive product for the PCR  
202 2 primer set; negative for PCR 1) compared with the untransfected wild-type (WT) parental line (PCR  
203 1 positive; PCR 2 negative). (c-h) Immunolocalisation was carried out on in vitro tachyzoite stages of  
204 the *TgGRA11B-HA3-mCHERRY* mutant (clone 1) at 24h post-inoculation. The exogenous TgGRA11B-  
205 HA3-mCherry was localised using rabbit anti-HA antibody (red; Stewart et al., 2016) and the mouse  
206 anti-recTgGRA11B antibody (green) in putative dense granules of tachyzoites using 0.25% Triton X-  
207 100 permeabilisation (c) and in the parasitophorous vacuole using a gentler 0.01% saponin  
208 permeabilisation (d). TgGRA11B-HA3-mCherry (red) co-localised with antisera to a bona fide dense  
209 granule protein, TgGRA1 (green; Stewart et al., 2016), in dense granules (0.25% Triton X-100, e) and  
210 in the parasitophorous vacuole (0.01% saponin, f). (g) Counterstaining with mouse anti-TgSAG1  
211 antibody (green; Stewart et al., 2016), distinguished the parasitophorous vacuole-staining of  
212 TgGRA11B-HA3-mCherry (red) from the tachyzoite surface (green; 0.01% saponin). (h) Similarly,  
213 native mCherry fluorescence from TgGRA11B-HA3-mCherry also confirmed dense granule  
214 localisation in early stages of tachyzoite expansion (first image), with parasitophorous vacuole  
215 localisation in later development (second and third images). Anti-HA and anti-TgGRA11B antibodies  
216 failed to stain tachyzoites of the wild-type parental strain and, likewise, no mCherry signal was  
217 detected in wild-type parental tachyzoites (data not shown). Scale bars are 2µm.

218