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1 HCF101 is a novel component of the CIA cytosolic iron-sulfur synthesis pathway  
2 in the human pathogen *Toxoplasma gondii*

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8

9 **Abstract**

10 Several key cellular functions depend on proteins harboring an iron-sulfur (Fe-S) cofactor. As these Fe-  
11 S proteins localize to several subcellular compartments, they require a dedicated machinery for  
12 cofactor assembly. For instance, in plants and algae there are Fe-S cluster synthesis pathways localizing  
13 to the cytosol, but also present in the mitochondrion and the in chloroplast, two organelles of  
14 endosymbiotic origin. *Toxoplasma gondii* is a plastid-bearing parasitic protist responsible for a  
15 pathology affecting humans and other warm-blooded vertebrates. We have characterized the  
16 *Toxoplasma* homologue of HCF101, originally identified in plants as a protein transferring Fe-S clusters  
17 to photosystem I subunits in the chloroplast. Contrarily to plants, we have shown that HCF101 does  
18 not localize to the plastid in parasites, but instead is an important component of the cytosolic Fe-S  
19 assembly (CIA) pathway which is vital for *Toxoplasma*. While the CIA pathway is widely conserved in  
20 eukaryotes, it is the first time the involvement of HCF101 in this pan-eukaryotic machinery is  
21 established. Moreover, as this protein is essential for parasite viability and absent from its mammalian  
22 hosts, it constitutes a novel and promising potential drug target.

## 23 Introduction

24 Iron-sulfur (Fe-S) clusters are ancient inorganic cofactors of proteins which are essential in virtually all  
25 forms of life [1]. These cofactors are found in a variety of proteins involved in numerous electron  
26 transfer and metabolic reactions that support key housekeeping cellular functions like respiration,  
27 photosynthesis, DNA repair and replication, protein translation, RNA modifications, but also in  
28 regulatory proteins and environmental signals sensors [2,3]. The most common clusters, which in most  
29 Fe-S proteins function as electron transfer groups [4], are in the rhombic [2Fe-2S], cubane [4Fe-4S], or  
30 more rarely the intermediate [3Fe-4S] configuration. Assembly of these cofactors is a carefully  
31 controlled process in order to avoid cellular toxicity arising from the accumulation of free Fe and S,  
32 which are highly reactive. Different Fe-S cluster assembly pathways developed early in evolution to  
33 synthesize the clusters and can be found in archaea, bacteria, and then evolved to mitochondrial,  
34 plastidial and cytosolic iron-sulfur assembly machineries in eukaryotes [5,6]. Thus, eukaryotic Fe-S  
35 cluster assembly machineries found in organelles of endosymbiotic origin were inherited from  
36 Alphaproteobacteria (the 'iron sulfur cluster', or ISC, pathway in the mitochondrion) and  
37 Cyanobacteria (the 'sulfur mobilization', or SUF, pathway in plastids), while a specific machinery  
38 ('cytosolic iron-sulfur cluster assembly', or CIA) is used for the biogenesis of cytosolic and nuclear Fe-S  
39 proteins.

40 While the individual components of these Fe-S assembly machineries display some structural diversity,  
41 they involve similar biochemical steps [7]. Typically, a cysteine desulfurase will generate sulfur from L-  
42 cysteine, scaffold proteins and chaperones will provide a molecular platform allowing the assembly of  
43 Fe and S into a cluster, and finally carrier proteins will deliver the cluster to target apoproteins. This  
44 happens in a similar fashion but with a specific molecular machinery in the three subcellular  
45 compartments in which Fe-S cluster synthesis takes place in eukaryotes, with main difference that the  
46 early step of CIA depends on the mitochondrial machinery. In fact, although de novo cluster assembly  
47 has been suggested to happen in the cytosol of mammalian cells [8], experimental evidence in a large  
48 variety of eukaryotes points to the requirement of mitochondrial ISC components, in particular the  
49 Nfs1 cysteine desulfurase, to generate a S precursor that will be translocated to the cytosol [9–11]. In  
50 mammals and yeast, P-loop NTPases NUBP1 and NUBP2 are the scaffolds for initial [4Fe-4S] Fe-S  
51 cluster assembly in the CIA system [12] and electrons are provided by DRE2, together with TAH18  
52 (ATR3 in plants) [13]. Noticeably, some eukaryotes, like plants, lack a NUBP2 homologue and the  
53 scaffolding complex is instead composed of a dimer of NBP35, the NUBP1 homologue [14]. NAR1, a  
54 protein containing two [4Fe-4S] clusters, then acts as the Fe-S carrier and associates with the CIA  
55 targeting complex (CTC) [15]. The CTC, that will recognize client apoproteins through direct  
56 interactions and mediate the insertion of the Fe-S cluster, typically comprises MET18/MMS19  
57 (plant/human nomenclature), CIA1/CIAO1, and AE7/CIAO2B [16].

58 *Toxoplasma gondii* is a widespread obligate intracellular parasitic protist belonging to the phylum  
59 Apicomplexa. Infection is usually harmless to immunocompetent individuals, but can lead to severe,  
60 life-threatening, disease in developing fetuses and immunocompromised individuals [17]. This parasite  
61 contains two organelles of endosymbiotic origin: a mitochondrion and a relict plastid called apicoplast,  
62 which are both of high metabolic importance [18]. Consequently, like in plants and algae *T. gondii*  
63 harbors three distinct Fe-S cluster synthesis pathways: the CIA in the cytosol, ISC in the mitochondrion  
64 and SUF in the apicoplast [19,20]. The SUF pathway being essential for the survival of the parasite and  
65 absent from its mammalian host, its components and client proteins are particularly attractive as  
66 potential drug targets [21], which prompted us to look for Fe-S proteins absent from mammals but  
67 present in plant and apicomplexan parasites. Investigating the Fe-S cluster assembly components in

68 protists, which are highly divergent phylogenetically from the canonical yeast or mammalian cell  
69 models, can bring interesting insights into the evolution of this molecular machinery [22,23].

70 Here, we report the characterization of the *T. gondii* homologue of the high-chlorophyll-fluorescence  
71 101 (HCF101) protein, which is typically a plastid-associated Fe-S transfer protein in the plant  
72 *Arabidopsis thaliana* [24,25]. Our work shows that TgHCF101 is essential for parasite viability and we  
73 demonstrate for the first time its implication in the eukaryotic CIA pathway.

74

## 75 **Results**

### 76 *TgHCF101 is a cytosolic protein*

77 HCF101 is a putative P-loop containing nucleoside triphosphate hydrolase that belongs to the  
78 Mrp/NBP35 family (also called ApbC) of dimeric iron-sulfur carrier proteins that are found ubiquitously  
79 in all domains of life and thus likely appeared early during evolution [26,27]. Compared with the  
80 NBP35, in addition to the nucleotide hydrolase domain HCF101 also contains a N-terminal  
81 DUF59/MIP18-family domain [28], as well as a C-terminal domain of unknown function (DUF971). We  
82 performed homology searches in the <http://www.ToxoDB.org> database to retrieve members of the  
83 Mrp/NPB35 family encoded by the *T. gondii* genome. Besides TgNBP35, that has an unusual  
84 mitochondrial localization in *T. gondii* [20,23], we identified TGGT1\_318590 as a potential homologue  
85 of HCF101. A broader phylogenetic analysis that included members of the Mrp/NPB35 family from a  
86 wide variety of eukaryotes (Table S1), revealed that this putative *T. gondii* homologue clearly  
87 segregates with members of the HCF101 group (Fig. 1A). Moreover, its primary sequence analysis  
88 confirmed a three domain-organization typical of HCF101 proteins (Fig. 2A), and hence TGGT1\_318590  
89 may be named TgHCF101.

90 In *A. thaliana*, the *HCF101* mutant was found to be impaired in photosynthesis [29]: interfering with  
91 HCF101 function has an impact on the maturation of both photosystem I (PSI) and ferredoxin-  
92 thioredoxin reductases, which contain [4Fe-4S] clusters [24,25]. The apicoplast harbored by  
93 apicomplexan parasites has lost the ability to perform photosynthesis [30], and some members of the  
94 Apicomplexa phylum, like *Cryptosporidium*, have even completely lost the plastid [31] while it has  
95 retained a HCF101 homologue (Fig. 1A, Fig. S1). Thus, a function linked to photosystem seems unlikely  
96 for apicomplexan parasites. Sequence analysis confirmed that, in contrast to plant homologues,  
97 TgHCF101 lacks a predicted N-terminal transit peptide, which is typically needed for plastid import (Fig.  
98 2B). Interestingly, *Chromera velia*, a close non-parasitic relative of Apicomplexa whose plastid has  
99 retained its photosynthetic capacity [32], has two HCF101 isoforms: one with a predicted transit  
100 peptide (potentially addressed to the plastid), and one without (Fig. 1, Fig. S1). A recent study suggests  
101 that the plastid-located HCF101 in fact evolved from an ancestral cytosolic HCF101 [23]. This led us to  
102 hypothesize that apicomplexan HCF101 would be involved in a cytosolic-related, rather than in a  
103 plastid-associated, Fe-S cluster transport function. Additional supporting evidence comes from the  
104 characterization of a C-terminal targeting complex recognition (TCR) signal ending with an aromatic  
105 residue ([LIM]-[DES]-[WF]) in the clients proteins of the cytosolic Fe-S cluster machinery or their  
106 adaptors [33], which can be clearly identified in the apicomplexan HCF101 homologues, as well as in  
107 the *C. velia* isoform without transit peptide (Fig. S1).

108 To assess experimentally the localization of TgHCF101, we first tagged an ectopic copy with a C-  
109 terminal GFP and observed a cytosolic localization (Fig. S2A), in line with what had been described  
110 previously when an additional copy of TgHCF101 bearing a C-terminal Human influenza hemagglutinin  
111 (HA) tag was expressed [23]. However, given the potential importance of the C-terminal TCR signal for

112 HCF101 function or localization, we next generated a transgenic cell line in which we modified the 5'  
113 region of the *TgHCF101* gene by homologous recombination to replace the endogenous promoter by  
114 an inducible-Tet07SAG4 promoter, and at the same time adding a sequence coding for a N-terminal  
115 hemagglutinin (HA) tag (Fig. 1C, D). In this conditional knock-down (cKD) HA-TgHCF101 cell line, the  
116 addition of anhydrotetracycline (ATc) can repress transcription of *TgHCF101* through a Tet-Off system  
117 [34]. Two independent transgenic clones were obtained and found to behave similarly in the initial  
118 phenotypic assays we performed, so only one will be described in details here.

119 Immunoblot analysis showed a N-terminal HA-tagged protein at around 70 kDa (the predicted  
120 molecular mass for TgHCF101), and depletion of the protein was efficient upon addition of ATc (Fig.  
121 1E). Immunofluorescence assay (IFA) showed a punctate cytosolic signal for TgHCF101, clearly distinct  
122 from the apicoplast, and efficiently depleted by the addition of ATc (Fig. 1F). Disruption of the  
123 apicoplast-located SUF pathway has a strong impact on the lipoylation of the E2 subunit of the  
124 pyruvate dehydrogenase (PDH-E2) by Fe-S-containing lipoate synthase LipA [19,21]. However,  
125 depletion of TgHCF101 did not seem to have an impact on the lipoylation profile of PDH-E2 (Fig. S2B).  
126 Disruption of the SUF pathway also typically impacts the apicoplast-located isoprenoid synthesis,  
127 which involves two Fe-S oxidoreductases (IspG and IspH) and has been shown to have downstream  
128 consequences on the glycosylation of proteins involved in parasite motility [21]. We thus assessed the  
129 gliding motility of the parasites after TgHCF101 depletion and found that it was only affected after four  
130 days of ATc treatment, which might be reflecting long-term impact of TgHCF101 depletion on parasite  
131 fitness rather than a direct consequence of apicoplast-related Fe-S cluster synthesis (Fig. S3A, B).

132 Altogether, our results indicate that TgHCF101 is not associated with the apicoplast-located SUF  
133 pathway, but localizes to the cytosol instead.

134

### 135 *TgHCF101 is essential for parasite growth*

136 We next assessed the impact of TgHCF101 depletion on the parasite's lytic cycle: we evaluated the  
137 capacity of TgHCF101-depleted tachyzoites (a highly replicative and highly invasive form responsible  
138 for the acute form of the disease caused by *T. gondii*) to generate lysis plaques. For this, we infected a  
139 monolayer of host cells in absence or continuous presence of ATc for 7 days (Fig. 2A, B). Depletion of  
140 TgHCF101 completely prevented plaque formation. While absence of plaques highlights a fitness  
141 problem for the parasites, it does not necessarily implies their death: for instance, in response to stress,  
142 tachyzoites can convert into a slow-growing and cyst-enclosed persisting stage called bradyzoites  
143 (associated with the chronic form of the disease), that can reactivate when they encounter more  
144 favourable conditions [35]. We thus performed a similar experiment, in which the ATc was washed out  
145 at the end of the 7-day incubation, and then incubated the parasites for an extra 7 days in the absence  
146 or presence of ATc before evaluating plaque formation (Fig. 2C). We did not observe any plaques after  
147 ATc removal, suggesting that the parasites were not able to recover after 7 days of TgHCF101  
148 depletion, likely because they were dead.

149 To assess whether this defect in the lytic cycle is due to a replication problem, cKD HA-TgHCF101  
150 parasites were preincubated in ATc for 48 hours and then released mechanically, before infecting new  
151 host cells for an additional 24 hours in ATc prior to parasite counting. We noted that incubation with  
152 ATc led to an accumulation of vacuoles with fewer parasites (Fig. 2D). To get more precise insights into  
153 the impact of TgHCF101 depletion on parasite division, we labelled dividing parasites with inner  
154 membrane complex (IMC) protein IMC3 [36] to detect growing daughter cells (Fig. 2E). *T. gondii*  
155 tachyzoites develop inside a mother cell by a process called endodyogeny [37] and this division is  
156 usually highly synchronous within the same parasitophorous vacuole, yet after two days of ATc

157 treatment vacuoles showed a marked lack of synchronicity for daughter cell budding. Investigation of  
158 the consequences of TgHCF101 depletion on developing parasites by electron microscopy highlighted  
159 important defects on cytokinesis, although the overall appearance of organelles seemed essentially  
160 normal (Fig. 2F). Finally, through propidium iodide labelling and flow cytometer-based analysis of DNA  
161 content, we observed that long-term depletion of TgHCF101 led to the emergence of an increasing  
162 subpopulation of parasites with sub-1N DNA content, thus smaller than the typical haploid 1N DNA  
163 content if the parasites (Fig. 2G), suggesting an impairment in DNA synthesis.

164 Overall, our results indicate that TgHCF101 depletion leads to important and, apparently, irreversible  
165 defects in parasite replication and growth.

166

### 167 *Depletion of TgHCF101 induces stress response mechanisms*

168 Through electron microscopy analysis, we also noticed that the depletion of TgHCF101 induced the  
169 appearance of structures resembling lipid droplets (LDs) in several parasites (Fig. 3A). In order to  
170 properly quantify this, we used Nile red, a selective fluorescent stain for neutral lipids, and microscopic  
171 imaging (Fig. 3B). We noticed a strong increase in both the number and size of LD in cKD HA-TgHCF101  
172 parasites incubated for two days with ATc (Fig. 3C, D). To see if this was a common feature induced by  
173 abolishing Fe-S synthesis, we quantified LDs in mutants of the mitochondrial (TgISU1) and apicoplast  
174 (TgSUFC) pathways [19]. Specific depletion of these proteins did not lead to an increase in LD formation  
175 (Fig. S4A, B), indicating it is specific to the depletion of TgHCF101. To assess if the induction of LD  
176 formation could play a role in the demise of the parasites, we used T863, a pharmaceutical inhibitor of  
177 the acyl coenzyme A:diacylglycerol acyltransferase 1 (DGAT1) enzyme, which is important for storing  
178 neutral lipids in cytoplasmic LD [38]. While treatment with T863 efficiently decreased the number of  
179 LDs (Fig. S4C, D, E), long term incubation during plaque assays did not allow any recovery in parasite  
180 fitness (Fig. S4F). This suggests that LDs are not detrimental to TgHCF101-depleted parasites but  
181 instead could be art of an integrated stress response, as they have been shown in other eukaryotes to  
182 be upregulated in response to cellular injuries including nutrient-related and oxidative stresses [39].

183 Disrupting proteins involved in Fe-S cluster assembly may have a direct effect on the stability and  
184 expression levels of local Fe-S proteins. Thus, to get insights into the effect of TgHCF101 depletion at  
185 the molecular level, we performed global label-free quantitative proteomic analyses. Of course, this  
186 may also affect downstream cellular pathways or functions, and other pathways may also be  
187 upregulated in compensation. cKD HA-TgHCF101 or TATI  $\Delta$ Ku80 parental control parasites were  
188 treated for three days with ATc prior to a global proteomic analysis and compared for protein  
189 expression. We selected candidates with a  $\log_2(\text{fold change}) \leq -0.55$  or  $\geq 0.55$  (corresponding to a  $\sim 1.47$ -  
190 fold change in decreased or increased expression) and a p-value  $< 0.05$  (ANOVA,  $n=4$  biological  
191 replicates) and we completed this dataset by selecting some candidates that were consistently and  
192 specifically absent from the mutant cell lines or only expressed in these (Tables S2 and S3). Many  
193 higher-expressed proteins were bradyzoite stage-specific and more particularly components of the  
194 cyst wall or their cell surface [40,41] (Fig. 4A, B, Table S2). Tachyzoites can convert to the persistent  
195 bradyzoite form upon stress, so to check if TgHCF101 depletion was inducing stage conversion we used  
196 a lectin from the plant *Dolichos biflorus*, which recognizes the SRS44/CST1 cyst wall glycoprotein in  
197 differentiating cysts [42] (Fig. 4C, D). We could see that TgHCF101 depletion induced the appearance  
198 of up to 10% of lectin-labelled vacuoles during the first 48 hours of intracellular growth in the presence  
199 of ATc. However, when parasites were kept for up to a week in the presence of ATc, the proportion of  
200 lectin-positive vacuoles plateaued at 14% and only a very limited number of vacuoles looked like bona



201 fide mature cysts (less than 2% were both lectin-positive and containing more than 2 parasites) (Fig.  
202 4E).

203 These data suggest that depletion of TgHCF101 leads to a cellular stress that initiates stage conversion  
204 into the bradyzoite stage, but that the differentiation process into mature cysts cannot be completed.

205

#### 206 *Depletion of TgHCF101 affects specifically cytosolic and nuclear Fe-S proteins*

207 The label-free quantitative proteomic analyses also highlighted proteins which were less expressed in  
208 absence of TgHCF101. Among them, were a surprisingly large number of rhoptry bulb proteins (Table  
209 S3, Fig. S5A). Rhoptries are club-shaped organelles that comprise a narrow tubular neck opening at the  
210 anterior pole of the parasite, and a most posterior bulbous part; the proteins secreted from these  
211 different sub-compartments are either involved in invasion and parasitophorous vacuole formation,  
212 or in the modulation of host cell defenses, respectively [43]. We assessed if there was any major impact  
213 of TgHCF101 depletion on rhoptry morphology or function. We performed IFA with anti-armadillo  
214 repeats only protein (TgARO), a protein homogenously anchored to the surface of the rhoptry  
215 membrane [44], and found no particular defect in rhoptry morphology or positioning (Fig. S5B).  
216 Quantification of intracellular evacuoles (rhoptry-secreted vesicular clusters) did not indicate any  
217 particular problem in the secretory capacity of the organelles upon TgHCF101 (Fig. S5C). Finally, we  
218 assessed by immunoblot the expression profile of one of the potentially less-expressed rhoptry bulb  
219 protein but found only a slight alteration in the expression profile (a modest increase in the non-  
220 mature form of the protein), but not in the overall amount of protein (Fig. S5D). We conclude that  
221 although TgHCF101 depletion may lead to a secondary impact on rhoptry content, it does not  
222 massively impact organelle morphology or secretory function.

223 Given the potential implication of TgHCF101 in the biogenesis of Fe-S proteins, we also specifically  
224 searched for putative Fe-S proteins in the less expressed proteins highlighted by the label-free  
225 quantitative proteomic analysis. We have previously estimated the Fe-S proteome using a  
226 computational predicting metal-binding sites in protein sequences [45], which, coupled with the data  
227 from global mapping of *T. gondii* proteins subcellular location by HyperLOPIT spatial proteomics [46],  
228 allows predicting client proteins present in the mitochondrion, apicoplast or cytosolic [19]. We found  
229 three Fe-S proteins that were less expressed upon TgHCF101 depletion (Fig. 5A). Interestingly, they  
230 were all connected to the CIA pathway as they were NAR1, a Fe-S carrier to the CIA core machinery  
231 [15], and two Fe-S proteins depending on the CIA machinery [47]: ABCE1 (Rli1 in yeast) a cytosolic  
232 ribosomal recycling factor and NTHL1, a nuclear DNA base-excision repair enzyme. In order to verify  
233 the proteomics data, we tagged TgABCE1 with a myc epitope in the context of the cKD HA-TgHCF101  
234 mutant (Fig. S6A, B). IFA showed a decrease in the cytosolic TgABCE1 signal (Fig. 5B), which was  
235 supported by quantitative immunoblot analysis indicating a significant decrease in TgABCE1 upon  
236 treatment of cKD HA-TgHCF101 mutant with ATc (Fig. 5C, D).

237 These results indicate that perturbing TgHCF101 expression has consequences on proteins of the CIA  
238 machinery or depending on this machinery for the assembly of their Fe-S cluster.

239

#### 240 *TgHCF101 is likely a Fe-S transfer protein of the CIA complex.*

241 To get further insights on the role played by TgHCF101 in the CIA machinery, we next performed co-  
242 immunoprecipitations (co-IPs) and mass spectrometry identification of associated proteins, comparing  
243 lysates of cKD HA-TgHCF101 parasites grown in the presence of ATc or not (Table S4). Strikingly, we



244 pulled-down all three *T. gondii* homologues of the core components of the CTC: MET18, CIA1 and AE7  
245 (a MIP18 family protein) (Fig. 6A). We managed to tag the TgCIA1 protein with a myc epitope tag in  
246 the context of the HA-tagged TgHCF101 cell line (Fig. S6C, D) and performed a reverse IP by which we  
247 managed to specifically pull-down TgHCF101, confirming their interaction (Fig. 6B). It should be noted  
248 that this result was only obtained when this experiment was performed after chemical crosslinking,  
249 suggesting a possibly indirect or transient interaction between the proteins.

250 Another interesting candidate that was found co-immunoprecipitated with TgHCF101 was TgABCE1,  
251 strengthening the possibility of an important relationship between the two proteins. We next  
252 performed a reverse IP and could recover small amounts of TgHCF101 co-eluting specifically with  
253 TgABCE1 (Fig. 6C). To further investigate this potential interaction, we performed yeast two-hybrid  
254 assay, based on interaction-dependent transactivation of the HIS3 reporter gene (Fig. 6D). This  
255 experiment confirmed that there is a direct interaction between TgHCF101 and TgABCE1, and  
256 highlighted a particularly strong interaction, as it was retained when using 3-aminotriazole, a  
257 competitive inhibitor of the HIS3 gene product (Fig. 6D). This confirms that TgABCE1 is likely a client  
258 protein of TgHCF101. Given the known implication of ABCE1 in translation, we next evaluated the  
259 impact of TgHCF101 depletion on translation by using a puromycin-based assay (Fig. 6E). Puromycin  
260 can mimic aminoacyl-tRNA and becomes covalently attached to nascent peptides, thereby allowing  
261 the evaluation of protein elongation rates with an anti-puromycin antibody [48]. Using this method,  
262 we could show that TgHCF101 depletion leads to a modest but a significant decrease in the overall  
263 translation rate (Fig. 6E, F).

264 Together, these results suggest that TgHCF101 acts as a Fe-S transfer protein to the translation  
265 regulator TgABCE1.

266

## 267 Discussion

268 Fe-S clusters are universal among living organisms, where they play key roles in many important  
269 biological processes as cofactors of proteins involved for instance in housekeeping functions like  
270 respiration, photosynthesis, as well as genome expression or maintenance [6]. These cofactors were  
271 acquired early during evolution, and thus they played a fundamental role in the evolution of the  
272 eukaryotic cells as they were inherited through endosymbiosis to organelles such as the mitochondrion  
273 or plastids [5]. Different Fe-S cluster synthesis pathways show globally-conserved mechanistic and  
274 biochemical features to assemble and transfer the clusters prior to transferring them to client proteins  
275 [7]. However, beyond a core Fe-S synthesizing machinery, given the large diversity of eukaryotic  
276 lineages, it is likely that some have evolved some specific features.

277 For instance proteins of the MRP/NBP35 family (NBP35, Cfd1, Ind1 and HCF101), have in common a  
278 central P-loop NTPase domain [26], but are either involved in scaffolding or transfer of Fe-S clusters at  
279 specific sub-cellular locations like the mitochondrion, plastid, or in the cytosol [12,24,25,49]. Presence  
280 of MRP family members in bacteria that are able to bind Fe-S clusters point to ancient and conserved  
281 function linked to Fe-S biogenesis for these proteins [50]. Another feature shared by members of this  
282 family is their ability to form homo- or hetero-dimers bridging one Fe-S cluster at their interface  
283 through two conserved and functionally essential Cys residues [51]. Interestingly, HCF101 also presents  
284 at its N-terminal a MIP18-like domain that constitutes most of a bacterial protein called SufT that is  
285 involved in Fe-S maturation [28,50,52], as well as a C-terminal domain whose function is not yet  
286 defined (DUF971). The presence of additional domain besides the conserved NTPase core domain in  
287 members of the MRP family like HCF101 suggests a potential complexification linked to more  
288 specialized functions. Another clue of evolutionary specialization comes from the different localization

289 of protein isoforms in different species, hinting for a possible association to different Fe-S cluster  
290 biogenesis machineries. While HCF101 was initially characterized as a chloroplast-associated protein  
291 with a specialized function in maintaining PSI homeostasis, a recent study suggested a wider diversity  
292 in localization for HCF101 paralogs across eukaryotes, especially in alveolates, with isoforms  
293 potentially localizing to the mitochondrion in *Tetrahymena* or in the cytosol in *T. gondii* [23]. Our study  
294 confirms that although *T. gondii* harbors a plastid, TgHCF101 is expressed in the cytosol (Fig. 1F, Fig.  
295 S2B), which is consistent with the absence of photosynthesis-related pathways in *T. gondii*. Our study  
296 establishes for the first time that the cytosolic expression of TgHCF101 is functionally related to the  
297 CIA pathway (Fig. 7).

298 Previous functional investigations of the CIA pathway in *T. gondii* focused on TgABCB7, a mitochondrial  
299 transporter presumably involved in providing a sulfur-containing precursor for further processing by  
300 the cytosolic Fe-S assembly machinery [53] and on TgNBP35, a Fe-S scaffolding protein of the CIA that  
301 displays an unusual association with the outer mitochondrial membrane in the parasites [20]. These  
302 two proteins are quite upstream in the CIA machinery, and imply that the assembly of cytosolic Fe-S  
303 clusters likely happens at the cytosolic face of the mitochondrion in *T. gondii*, and then probably shuttle  
304 through the *T. gondii* NAR1 homologue to the CTC for subsequent transfer to client proteins (Fig. 7).  
305 CTC proteins have so far not been functionally investigated in the parasite, but both TgABCB7 and  
306 TgNBP35 are essential for parasite growth and were both shown to be important for TgABCE1 stability  
307 [20,53]. Combining genome-wide data for potential essentiality and localization [46,54] to metal-  
308 binding site prediction algorithms [45], our previous assessment of the putative *T. gondii* cytosolic and  
309 nuclear Fe-S proteome highlighted proteins involved in key functions like genome reparation and  
310 maintenance, mRNA synthesis and protein expression [19]. It is thus unsurprising to see that *T. gondii*  
311 mutants of the CIA pathway are severely impaired in growth. Our quantitative proteomics analysis of  
312 the TgHCF101 mutant did not reveal an impact on many nuclear or cytosolic Fe-S proteins, which may  
313 indicate that this protein could be involved in cluster transfer to a reduced subset of Fe-S proteins.  
314 Besides the decrease in abundance of TgNAR1, strengthening the evidence of TgHCF101 involvement  
315 in the CIA pathway, quantitative proteomics data revealed a decrease in two potential client Fe-S  
316 proteins: TgABCE1 and TgNTHL1. While we could establish a direct association of TgHCF101 with  
317 TgABCE1 by co-immunoprecipitations and yeast two hybrid (Fig. 6A, C, D), we did not manage to tag  
318 TgNTHL1 to perform co-immunoprecipitations and did not obtain proof of a direct TgHCF101/TgNTHL1  
319 interaction by yeast two hybrid.

320 Although it is not firmly established that TgNTHL1 is a client protein of TgHCF101, our phenotypic  
321 analysis of TgHCF101-depleted parasites revealed cell division and DNA replication problems (Fig. 2D,  
322 G) that might result from an impact on Fe-S protein-mediated DNA maintenance. In addition, through  
323 perturbation of TgABCE1 function, we measured a decrease in the overall translation rate in the  
324 parasites (Fig. 6E, F), that could contribute to their decreased fitness and growth problems. Upon two  
325 days of TgHCF101 depletion, parasites also started to display an accumulation of LDs (Fig. 3). LDs are  
326 known to be induced as part of an integrated stress response to cellular injuries and may be induced  
327 in conditions such as oxidative stress [39]. For instance, during the particular Fe-induced cell death  
328 called ferroptosis iron excess, combined to oxygen, leads to the accumulation of reactive oxygen  
329 species (ROS), leading to subsequent peroxidation of lipids, which in turn induces the accumulation of  
330 lipid droplets that act as antioxidant organelles to control polyunsaturated fatty acid storage in  
331 triglycerides in order to reduce membrane lipid peroxidation [55]. Although it is possible that  
332 perturbation of the CIA pathway created local imbalance in cytosolic Fe concentration and ROS-  
333 dependent stress that contributed to the demise of the parasites, it is to note that our previous work  
334 has shown that general Fe deprivation has a similar LD-inducing effect on the parasites [56]. So,  
335 altogether this may rather suggest that specific CIA-dependent Fe-S protein(s) that remain to be

336 determined could be involved in regulating LDs. Even more so than the induction of LDs, initiation of  
337 stage conversion to the bradyzoite persistence form is a hallmark of the stress response in *T. gondii*  
338 [35]. Upon depletion of TgHCF101 we could detect early signs of differentiation (Fig. 4), but the  
339 parasites were largely unable to progress to full stage conversion and died instead (Fig. 2C, Fig. 4E).  
340 Importantly, overall our data show that TgHCF101 is essential for parasite viability, and although our  
341 findings point towards an involvement of this protein in the eukaryote-conserved CIA pathway, its  
342 specific absence from the mammalian hosts of the parasite makes it a good potential drug target. This  
343 calls for further structure/function studies of TgHCF101 in order to potentially design specific  
344 inhibitors.

345 There is a vast repertoire of Fe-S client proteins with a diverse range of structures and roles, and one  
346 important conundrum remaining in the Fe-S research field is resolving functional specificity of the  
347 transfer proteins during the addition of Fe-S to apoproteins. Key investigations in budding yeast  
348 allowed the identification of mutually exclusive sub-complexes of the CTC that can transfer Fe-S  
349 clusters to different proteins [47,57]. In particular, it was shown that a complex of two small proteins,  
350 Yae1 and Lto1, functions as a target-specific adaptor to recruit the yeast homologue of ABCE1 (called  
351 Rli1) to the generic CIA machinery [57]. These adaptor proteins are well-conserved in phylogenetically-  
352 close opisthokonts like humans, in which they have also been shown to be important for ABCE1  
353 maturation [58,59]. However, Yae1 and Lto1 are clearly not conserved across all eukaryotes but found  
354 essentially in fungi, metazoan and plants (Fig. S7), and thus noticeably they do not seem to have  
355 homologues in the Alveolata superphylum that includes apicomplexan parasites. So, while ABCE1 is  
356 arguably one of the most conserved proteins in evolution and is universally present in all eukaryotes  
357 [60], the factors driving its CIA-dependent maturation seem phylogenetically divergent. We have now  
358 established that in *T. gondii*, HCF101 is associated to the CTC and likely plays that role in this organism.  
359 Given sequence conservation of HCF101 homologs, it is also likely to be the case in other apicomplexan  
360 parasites and even possibly in one isoform present in the photosynthetic relative *C. velia* (Fig. 1A, B,  
361 Fig. S1). Our results highlight the complex evolutionary adaptation that accompanied the maturation  
362 of highly complex eukaryotic Fe-S protein and calls for in-depth structural analysis of this interaction.  
363 Not only this would provide invaluable evolutionary insights into the molecular machinery supporting  
364 Fe-S cluster transfer, but also may provide new prospects for interfering specifically with an essential  
365 function, as a new strategy against apicomplexan-caused diseases.

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## 371 **Methods**

### 372 *Cell culture*

373 *Toxoplasma gondii* RH tachyzoites and derived transgenic cell lines generated in this study were  
374 routinely maintained through passages in human foreskin fibroblasts (HFFs) monolayer (ATCC CRL-  
375 1634). HFFs and parasites were cultured in standard Dulbecco's Modified Eagle's Medium (DMEM,  
376 supplemented with 5% decompemented fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL  
377 penicillin and 100 µg/mL streptomycin (Gibco) in a controlled atmosphere at 37°C and with 5% CO<sub>2</sub>.  
378 For immunoprecipitation assays, parasites were grown in Vero cells (ATCC, CCL-81) in the same  
379 conditions as for the HFFs.

380 Transgenic cell lines were generated in the TATi  $\Delta$ Ku80 genetic background line lacking the *Ku80* gene  
381 and expressing the TATi transactivator required for the TetOff system [61]. For positive selection of  
382 transgenic parasites bearing resistance cassettes for expressing dihydrofolate reductase thymidylate  
383 synthase (DHFR-TS) or chloramphenicol acetyltransferase (CAT) were grown with 1  $\mu$ M pyrimethamine  
384 or 20  $\mu$ M chloramphenicol (Sigma-Aldrich, SML3579, C0378), respectively. Conditional depletion in the  
385 TetOff conditional knockdown lines was achieved by incubation with 0,5  $\mu$ g/mL anhydrotetracycline  
386 (ATc, Fluka 37919) for the indicated duration of the assay.

387

#### 388 *Bioinformatic analyses*

389 Genomic and protein sequences were retrieved from <http://www.toxodb.org> or  
390 <https://www.ncbi.nlm.nih.gov/protein/>. Protein alignments were performed from eukaryotic MRP  
391 family proteins (Table S1), whose sequences were obtained from Grosche et al. [62], and updated by  
392 manual curation using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm of  
393 the Geneious software suite v 6.1.8 (<http://www.geneious.com>). Phylogenetic trees were constructed  
394 using the MEGA software v11.0.13 (<https://www.megasoftware.net/>), using the unweighted paired  
395 group mean arithmetic (UPGMA) method, with Poisson model for amino acid substitution.

396 Transit peptide and sub-cellular localization predictions were performed using the ChloroP 1.1  
397 (<https://services.healthtech.dtu.dk/services/ChloroP-1.1/>), IPSORT (<http://ipsort.hgc.jp/>), and  
398 Localizer 1.0.4 (<http://localizer.csiro.au/>) algorithms. Domain searches were performed using the  
399 Interpro database (<https://www.ebi.ac.uk/interpro/>).

400

401

#### 402 *Generating a GFP-tagged TgHCF101 cell line*

403 cDNA corresponding to the *TgHCF101* gene (TGGT1\_318590) was amplified by PCR with primers  
404 ML4715 and ML4716 (all primers used in the present study are listed in Table S5) and sub-cloned using  
405 XhoI and KpnI into the pEZS-NL vector (D. Ehrhardt,  
406 <https://deepgreen.dpb.carnegiescience.edu/cell%20imaging%20site%20/html/vectors.html>) for a C-  
407 terminal GFP fusion. The *TgHCF101*-GFP cassette was then amplified by PCR using the ML4817 and  
408 ML4818 primers and cloned using BclI and EcoRV in a BglII/EcoRV-digested pTUB-IMC1-TdT vector [63]  
409 to drive the expression from a tubulin promoter. Tachyzoites were transfected with 100  $\mu$ g of plasmid  
410 and observed by fluorescence microscopy.

411

#### 412 *Generating a conditional TgHCF101 knock-down cell line*

413 To generate the construct for the tetracyclin-regulated conditional depletion of *TgHCF101* and add a  
414 N-terminal HA tag, we digested the DHFR-TetO7Sag4 plasmid [63] by BglII and inserted a fragment  
415 coding for a single HA tag generated through hybridization of the ML4924 and ML4925  
416 oligonucleotides. Then, a fragment corresponding to the 5' coding part of *TgHCF101* was amplified by  
417 PCR with primers ML4926 and ML4927 and inserted after digesting with BglII and NotI to yield the  
418 DHFR-TetO7Sag4-*TgHCF101* plasmid. The TATi  $\Delta$ Ku80 cell line was transfected with 80  $\mu$ g of the BsiWI-  
419 linearized DHFR-TetO7Sag4-*TgHCF101* plasmid. Transgenic parasites, named cKD HA-*TgHCF101*, were  
420 selected with pyrimethamine and cloned by limiting dilution. Positive clones were verified by PCR with  
421 primers ML1771 and ML6043.

422

#### 423 *Tagging of ABCE1 and CIA1 in the TgHCF101 knock-down background*

424 A CRISPR-based strategy was used to C-terminally tag proteins of interest in the cKD HA-*TgHCF101*  
425 background. Guide RNAs (gRNAs) targeting the C-terminal end of the gene of interest were selected  
426 using CHOP-CHOP tool (<https://chopchop.cbu.uib.no/>). gRNAs were cloned into the pU6-Cas9  
427 Universal Plasmid (Addgene, 52694) using BsaI. Donor DNA was amplified by PCR using the high fidelity  
428 KOD DNA polymerase (Novagen) to amplify a fragment containing the tag and the resistance cassette,  
429 using the pLIC-myc-CAT plasmid as a template, adding at both ends 30-nucleotide overhangs  
430 homologous to the C-terminal region of the gene of interest for homologous recombination. *TgABCE1*  
431 (TGGT1\_216790) tagging was performed by using primers ML5126 and ML5127 for the gRNA and



432 ML5128 and ML5129 to amplify donor DNA, yielding the cKD HA-TgHCF101 TgABCE1-myc cell line.  
433 Integration of the construct was controlled by PCR using GoTaq DNA polymerase (Promega) with  
434 primers ML6115 and ML4310. *TgCIA1* (TGGT1\_313280) tagging was performed by using primers  
435 ML5932 and ML5933 for the gRNA and ML5934 and ML5935 to amplify donor DNA, yielding the cKD  
436 HA-TgHCF101 TgCIA1-myc cell line. Integration of the construct was controlled by PCR using GoTaq  
437 DNA polymerase (Promega) with primers ML5936 and ML4310.

438

#### 439 *Immunofluorescence assays*

440 For immunofluorescence assays (IFA), coverslips seeded with HFFs were infected by *T. gondii*  
441 tachyzoites. Intracellular parasites and host cell monolayer were then fixed with 4% (w/v)  
442 paraformaldehyde (PFA, diluted in phosphate-buffered saline -PBS-) for 20 minutes. After fixation, cells  
443 were permeabilized with 0.3% (v/v) Triton X-100 (diluted in PBS) for 10 minutes. Coverslips were  
444 blocked with 2% (w/v) bovine serum albumin (BSA) for 1h prior to immunolabelling with primary  
445 antibody for 1 hour. After 3 washes in PBS, corresponding secondary antibody was incubated for 1  
446 hour. Coverslips were finally incubated with 1 µg/mL 4,6-diamidino-2-phenylindole (DAPI) for 5  
447 minutes before 3 washes in PBS and, lastly, mounted using Immu-Mount (ThermoFisher) onto  
448 microscope slides. Primary antibodies used were prepared in 2% BSA (diluted in PBS) and used at the  
449 following concentrations: rat monoclonal anti-HA (1:1000, 3F10 Roche), mouse monoclonal anti-myc  
450 (1:100, 9E10 Sigma), rabbit anti-IMC3 (1:1000) [64], mouse anti-SAG1 (T41E5, 1:1000) [65], rabbit anti-  
451 armadillo repeats only (ARO, 1:1000) [44]. Cysts were stained with biotin labelled *Dolichos Biflorus*  
452 lectin (1:300, Sigma L-6533) and detected with FITC-conjugated streptavidin (1:300, Invitrogen,  
453 SNN1008). Lipid droplets were detected with Nile Red (1 µg/mL, Sigma 72485).

454 All images were acquired at the Montpellier Ressources Imagerie (MRI) facility. Observations were  
455 performed with a Zeiss AxioImager Z1 epifluorescence microscope equipped with a Zeiss AxioCam  
456 MRm CCD camera and 63X/1.4 or 100X/1.4 Oil Plan Achromat objective. Images were processed on  
457 Zen Blue v3.6 (Blue edition) software (Zeiss). Z-stack acquisitions were processed by maximum  
458 intensity orthogonal projection when assessing lipid droplets number and area. Adjustments of  
459 brightness and contrast were applied uniformly and paired images were acquired with the same  
460 exposure time.

461

#### 462 *Plaque and replication assays*

463 The lytic cycle of tachyzoites was assessed by plaque assay as described previously [56]. Briefly,  
464 tachyzoites from the cKD HA-TgHCF101 transgenic cell line or parental strain (TATi ΔKu80) were  
465 allowed to invade monolayers of HFFs in the presence or absence of ATc. Parasites were cultivated for  
466 7 days at 37°C and 5% CO<sub>2</sub> and fixed with 4% (w/v) PFA (diluted in PBS) for 20 minutes. Cells were  
467 stained with 0.1% crystal violet solution (V5265, Sigma-Aldrich), washed and air-dried before imaging  
468 on an Olympus MVX10 microscope. For the reversibility assay, drug washout was carefully performed  
469 after 7 days of ATc pre-treatment and cultures were kept for another 7 days of growth, non-treated  
470 control conditions were also infected at this timepoint.

471 For replication assay, parasites were pre-treated in flasks for 48 hours with ATc and extracellular  
472 parasites were allowed to invade HFF monolayer on coverslips for another 24 hours before being fixed  
473 and performing parasites immunodetection by IFA with mouse anti-SAG1 antibody as described  
474 before. The number of parasites per vacuole was scored. Independent experiments were conducted  
475 three times, and 200 vacuoles were counted for each condition.

476

#### 477 *Immunoblotting and antibodies*

478 Protein extracts were prepared from 10<sup>7</sup> extracellular parasites resuspended in Laemmli buffer at a  
479 final concentration of 10<sup>6</sup> parasites/µL. Extracts were treated with benzonase to remove DNA from  
480 samples and resolved by SDS-PAGE before being transferred on nitrocellulose membrane for  
481 subsequent protein detection. Primary antibodies used for immunodetection were resuspended at  
482 their respective working concentration in 5% (w/v) milk in TNT buffer (0.1 M Tris-HCl, pH 7.6, 0.15 M  
483 NaCl, 0.05%, Tween 20). Antibodies used in this study for immunoblot detection were rat anti-HA

484 (1:1000, Roche), mouse anti-myc (1:100, Sigma), mouse anti-SAG1 (1:50, hybridoma), mouse anti-actin  
485 (1:25, hybridoma) [66], mouse anti-ROP7 (1:1000, T43H1) [67], mouse anti-puromycin (1:1000, 12D10,  
486 MABE943 Sigma) and rabbit anti-lipoic acid (1:500, ab58724 Abcam).

487

#### 488 *Puromycin labelling*

489 Strains of interest were grown for 3 days in the presence or absence of ATc. Freshly egressed parasites  
490 were filtered on 40µm Cell Strainer (VWR, 723-2757). After filtration and counting, parasites were  
491 treated with puromycin (100 µg/mL, puromycin dihydrochloride, Sigma) for 15 minutes at 37°C and  
492 5% CO<sub>2</sub>. For translation inhibition control, parasites were treated with cycloheximide (100 µg/mL,  
493 Sigma) for 10 minutes prior to puromycin incubation. After treatment, parasites were washed in DPBS  
494 (Dulbecco's phosphate-buffered saline, Gibco) and collected by centrifugation. Pellet was resuspended  
495 in Laemmli buffer and separated on Mini-Protean TGX Stain-free gels 12% (BioRad) activated by a UV-  
496 induced 1-minute reaction to produce tryptophan residue fluorescence in order to allow for global  
497 protein quantification, following manufacturer instructions. Proteins were then transferred to  
498 nitrocellulose membrane for immunodetection using mouse anti-puromycin (1:1000, 12D10, MABE943  
499 Sigma) and mouse anti-actin (1:25, hybridoma). Total protein content is assessed by Stain-free  
500 detection and puromycin signal is detected with secondary antibody mouse coupled with alkaline  
501 phosphatase. Both signals were quantified by densitometry using the ImageJ software.

502

#### 503 *Electron microscopy*

504 Parasites were pretreated with ATc for 48h and allowed to reinvade for 24h in the presence of ATc.  
505 Untreated parasites were used as a control for normal morphology. Cells were then fixed with 2.5%  
506 glutaraldehyde in cacodylate buffer 0.1 M pH7.4. Coverslips were subsequently processed using a  
507 Pelco Biowave pro+ (Ted Pella). Samples were postfixed in 1% OsO<sub>4</sub> and 2% uranyl acetate, dehydrated  
508 in acetonitrile series and embedded in Epon 118 using the following parameters: Glutaraldehyde (150  
509 W ON/OFF/ON 1-min cycles); two buffer washes (40 s 150 W); OsO<sub>4</sub> (150 W ON/OFF/ON/OFF/ON 1-  
510 min cycles); two water washes (40 s 150 W); uranyl acetate (100 W ON/OFF/ON 1-min cycles);  
511 dehydration (40 s 150 W); resin infiltration (350 W 3-min cycles). Fixation and infiltration steps were  
512 performed under vacuum. Polymerization was performed at 60°C for 48 hr. Ultrathin sections at 70  
513 nM were cut with a Leica UC7 ultramicrotome, counterstained with uranyl acetate and lead citrate and  
514 observed in a Jeol 1400+ transmission electron microscope from the MEA Montpellier Electron  
515 Microscopy Platform. All chemicals were from Electron Microscopy Sciences, and solvents were from  
516 Sigma.

517

#### 518 *Label-free quantitative proteomics*

519 Parasites from the TATi ΔKu80 and cKD HA-TgHCF101 cell lines were treated for 72 hours in HFF seeded  
520 in T75 cm<sup>2</sup> flasks. After suitable incubation, parasites were released from host cells with a cell scrapper  
521 and passed through a 25G needle before filtration on a fiber glass wool column. Parasites were pelleted  
522 and washed in Hank's Balanced Salt Solution (HBSS, Gibco). Parasites were resuspended in lysis buffer  
523 (1% SDS, 50mM Tris HCL pH 8, 10mM EDTA pH 8) and protein quantification was determined with the  
524 bicinchoninic acid kit (Abcam). For each condition, 20 µg of protein resuspended in Laemmli buffer  
525 were resolved on a 10% SDS-PAGE for 35 minutes at 100V. Proteins were fixed with a combination of  
526 acetic acid and ethanol and stained in PageBlue Protein Staining Solution (ThermoScientific). Each lane  
527 was cut in three identical pieces which were digested with trypsin and peptide extraction was done as  
528 previously described [68].

529 LC-MS/MS experiments were performed using an Ultimate 3000 RSLC nano system (ThermoFischer)  
530 interfaced online with a nano easy ion source and an Exploris 240 Plus Orbitrap mass spectrometer  
531 (ThermoFischer). The .raw files were analyzed with MaxQuant version 2.0.3.0 using default settings  
532 (PMID: 19029910). The minimal peptide length was set to 6. The files were searched against the *T.*  
533 *gondii* proteome (March 2020, <https://www.uniprot.org/proteomes/UP000005641-8450>). Identified  
534 proteins were filtered according to the following criteria: at least two different trypsin peptides with  
535 at least one unique peptide, an E value below 0.01 and a protein E value smaller than 0.01 were



536 required. Using the above criteria, the rate of false peptide sequence assignment and false protein  
537 identification were lower than 1%. Proteins were quantified by label-free method with MaxQuant  
538 software using unique and razor peptides intensities [69]. Statistical analyses were carried out using  
539 RStudio package software. The protein intensity ratio (protein intensity in mutant/protein intensity in  
540 parent) and statistical tests were applied to identify the significant differences in the protein  
541 abundance. Hits were retained if they were quantified in at least three of the four replicates in at least  
542 one experiment. Proteins with a significant ( $p < 0.05$  or  $0.01$  with or without Benjamini correction)  
543 quantitative ratio were considered as significantly up-regulated and down-regulated respectively.  
544 Volcano plots were generated with the Perseus software v2.0.7.0 (<https://maxquant.net/perseus/>).  
545 Perseus was also used for hierarchical clustering of bradyzoite- and tachyzoite-specific surface antigens  
546 of the SRS family using RNAseq data of Hehl et al. [70], available on [www.Toxodb.org](http://www.Toxodb.org).

547

#### 548 *Co-immunoprecipitation and mass spectrometry identification*

549 Parasites of the cKD HA-TgHCF101 transgenic cell line were treated for 3 days in the presence or  
550 absence of ATc in T175 cm<sup>2</sup> seeded with Vero cells. After treatment, intracellular parasites were  
551 released by scraping of the host cells and three passages through a 26G needle. To eliminate cell host  
552 debris, parasites were filtered through fiber glass wool and harvested by centrifugation at 650g for 5  
553 min and washed three times in DPBS (Gibco). Parasites were resuspended in lysis buffer (1%NP40, 50  
554 mM Tris-HCl pH8, 150 mM NaCl, 4 mM EDTA, supplemented with cOmplete Mini protease inhibitors  
555 mix (Roche)) and incubated overnight at 4°C on a rotating wheel. Centrifugation of insoluble material  
556 was performed at 13500g for 30 min at 4°C. The supernatant was transferred to a tube containing 50µL  
557 of anti-HA magnetic beads (ThermoFischer, 88836) for 4h at 4°C on a rotating wheel. The depleted  
558 fraction was then removed and beads were washed 5 times with lysis buffer. For the elution of  
559 immunoprecipitated proteins, beads were incubated in 50 µL of HA peptide solution at 2 mg/mL  
560 (ThermoFischer, 26184) for 1h at 37°C on a rotating wheel. The eluted proteins were mixed with  
561 Laemmli buffer and resolved on a 10% SDS-Page for 35 minutes at 100V. In-gel proteins were fixed  
562 with a combination of acetic acid and ethanol and stained with PageBlue Protein Staining Solution  
563 (ThermoScientific). Each lane was cut in three identical pieces which were digested with trypsin,  
564 peptide extraction was done as previously described [68] and mass spectrometry identification was  
565 performed as described for label-free quantitative proteomic.

566 Proteomic data were analyzed on R following the publicly available script of DEP analysis package  
567 (<https://github.com/arnesmits/DEP>, v.1.7.1). The following parameters were used for differential  
568 expression analysis: dataset was normalized by variance-stabilizing transformation and missing values  
569 were imputed via MNAR (missing not at random) method, the fold change cutoff was set at 1.5 and p-  
570 value at 0.05. Statistical analyses were performed by a differential enrichment test based on protein-  
571 wise linear models and empirical Bayes statistics [71] and p-values were adjusted by the Benjamini-  
572 Hochberg correction.

573 Validation of co-immunoprecipitation mass spectrometry results was performed by immunoblotting.  
574 C-terminally c-myc tagged candidates in the cKD HA-TgHCF101 background were grown in the presence  
575 or absence of ATc for 48h. Parasites were harvested and subjected to co-immunoprecipitation using  
576 myc-Trap agarose beads (Chromotek, yta-20) following manufacturer's instructions. Cross-linking of  
577 proteins was performed prior to co-immunoprecipitation for the cKD HA-TgHCF101 CIA1-myc cell line  
578 using 1% (w/v) PFA (diluted in PBS) for 5 minutes at room temperature and quenched with 125 mM  
579 ice-cold glycine. Immunoprecipitated proteins were then resolved on 10% SDS-Page prior to  
580 immunoblot analysis.

581

#### 582 *Yeast two-hybrid*

583 RNA was extracted from parasites using NucleoSpin RNA kit (Macherey-Nagel). cDNA was obtained  
584 using SuperScript III First Strand Synthesis SuperMix for RT-qPCR (Invitrogen) using oligodT and  
585 following the manufacturer's instructions. Specific cDNAs were subsequently amplified using KOD DNA  
586 polymerase (Novagen). Cloning was performed with In-Fusion HD cloning kit (Takara) for each  
587 candidate into pGADT7 and pGBKT7 vectors (Clontech, Takara Bio) to allow expression of AD-(Gal4

588 activation domain) and BD-(Gal4 DNA binding domain) fusion proteins, respectively. Primers were  
589 designed using the InFusion Cloning Primer Design Tool (<https://takarabio.com/>) and are listed in Table  
590 S5.

591 All experiments were performed in the Gal4-based yeast two-hybrid (Y2H) reporter strain YRG2  
592 (MAT $\alpha$ , *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *leu2-3*, *112*, *trp1-901*, *gal4-542*, *gal80-538*,  
593 *lys2::UAS<sub>GAL1</sub>-TATA<sub>GAL1</sub>-HIS3*, *URA3::UAS<sub>GAL4,17MERS(x3)</sub>-TATA<sub>CYC1</sub>-LacZ*) (Stratagene, Agilent). Yeast cells  
594 were co-transformed by pGAD/pGBK construct pairs and selected on plates containing the minimal  
595 YNB medium (0.7% Yeast Nitrogen Base medium without amino acids, 2% glucose, 2% Difco agar)  
596 supplemented with histidine (H), adenine (A), Lysine (K), and uracil (U) according to Gietz and Woods  
597 [72]. For all Y2H interaction assays, individual co-transformed colonies were cultured on liquid  
598 YNB+HUKA, adjusted to an OD<sub>600</sub> of 0.05 and dotted on YNB+UKA plates containing (+His plates) or  
599 missing (-His plates) histidine (7  $\mu$ L per dot). Plates were incubated at 30°C, and cell growth was  
600 recorded after 5 days. Constructs pairs including empty vectors (AD-fusion + empty BD, or empty AD +  
601 BD-fusion) were tested first to evaluate the capacity of each protein of interest to transactivate the  
602 *HIS3* reporter gene in the absence of interactor, and to determine the appropriate 3-aminotriazole  
603 (3AT) concentration required to abolish false interactions. True positive interactions were visualized  
604 as cells growing on YNB+UKA in the absence of histidine (-his plates) and eventually in the presence of  
605 3AT if required. Increasing 3AT concentrations were also used to challenge the strength of positive  
606 interactions. For all assays, at least three independent transformations per construct pairs were  
607 performed.

#### 608 *DNA content analysis*

609 DNA content analysis by flow cytometry was performed as described previously [73]. Parasites from  
610 the cKD HA-TgHCF101 strain and from the background line TATi  $\Delta$ Ku80 were treated for up to four days  
611 in the presence or absence of ATc. Extracellular parasites were removed by washing with HBSS and  
612 intracellular parasites were released from host cell by scraping with a cell scraper (VWR, 734-2602),  
613 passages through 26G needles and finally filtered on a fiber glass wool column. Parasites were then  
614 fixed overnight in a solution of 70% ethanol and 30% PBS at 4°C. After fixation, parasites were washed  
615 in PBS and stained with a 30  $\mu$ M propidium iodide solution for 30 min. Parasites DNA content was then  
616 analyzed by flow cytometry with an Aurora cytometer (Cytek) from the MRI facility.

#### 618 *Gliding motility assay*

619 Parasites from the parental TATi  $\Delta$ Ku80 and the cKD HA-TgHCF101 transgenic cell lines were grown in  
620 the presence or absence of ATc for up to 4 days.  $1.10^6$  freshly egressed parasites were harvested and  
621 resuspended in 400 $\mu$ L motility buffer (155 mM NaCl, 3 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 3 mM  
622 NaH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 10 mM glucose) and 100  $\mu$ L of the suspension was immediately placed on  
623 poly-L-lysine coated slides and incubated at 37°C for 15 min. Unattached parasites were removed by  
624 performing three washes with PBS. Finally, attached parasites were fixed with 4% (w/v) PFA and  
625 stained with anti-SAG1 antibody. Trails length were captured with a 63x objective on a Zeiss AXIO  
626 Imager Z2 epifluorescence microscope and were quantified using the NeuronJ plug-in on the ImageJ  
627 software as described previously [74].

#### 629 *Rhoptry secretion assay*

630 Rhoptry secretion was quantified by performing an evacuole assay [75]. Parasites from the cKD HA-  
631 TgHCF101 strain and cKD TgARO strain were grown in the presence or absence of tetracycline for up  
632 to 72 hours. Freshly egressed parasites were then pre-treated for 10 min in DMEM supplemented with  
633 1  $\mu$ M cytochalasin D (cytD), an inhibitor of actin polymerization. Parasites were allowed to secrete  
634 their rhoptry content as they were put in contact with HFF cells for 15 min in the presence of cytD at  
635 37°C. Parasites and HFF cells were then immediately fixed with 8% (w/v, in PBS) PFA, permeabilized  
636 with 0.1% Triton X-100 and stained with anti-ROP1 and anti-SAG1 to detect secreted rhoptry material  
637 and parasites, respectively. For each technical replicate, 20 fields were quantified and three technical  
638 replicates were performed for each of the three biological replicates.

640

#### 641 *Statistical analyses*

642 Statistical analyses were generally performed with the Prism 8.3 software (Graphpad). For proteomics  
643 experiments, statistics were performed with version 4.2.1 of the R package (2022-06-23,  
644 <https://www.R-project.org/>) using the Differential Enrichment analysis of Proteomics Data (DEP,  
645 v.1.23.0) package. Unless specified, values are expressed as means  $\pm$  standard deviation (SD).

646

#### 647 **Data availability**

648 All raw MS data and MaxQuant files generated have been deposited to the ProteomeXchange  
649 Consortium via the PRIDE partner repository (<https://www.ebi.ac.uk/pride/archive>) with the dataset  
650 identifiers PXD051549 and PXD051551.

651

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660

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664

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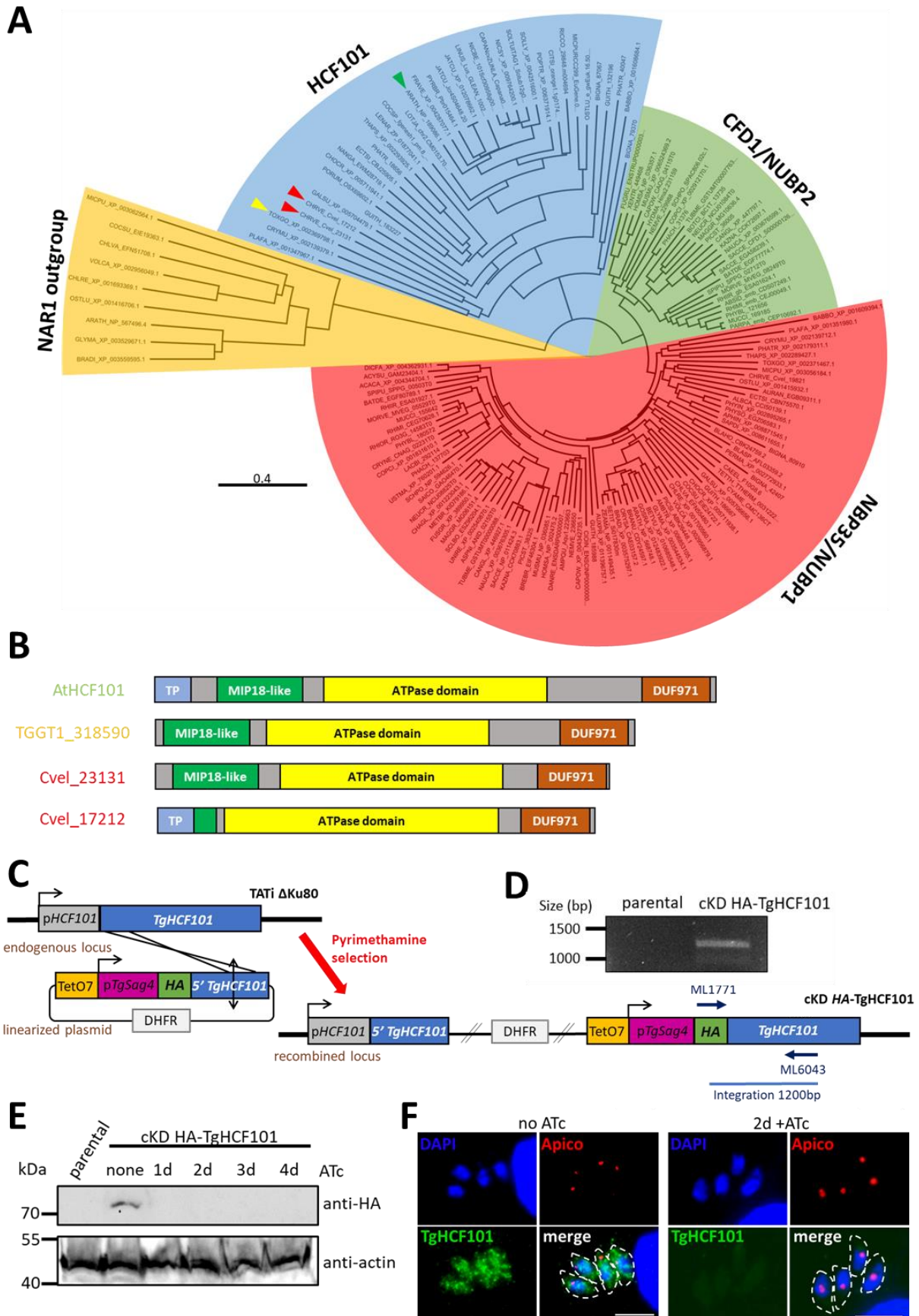
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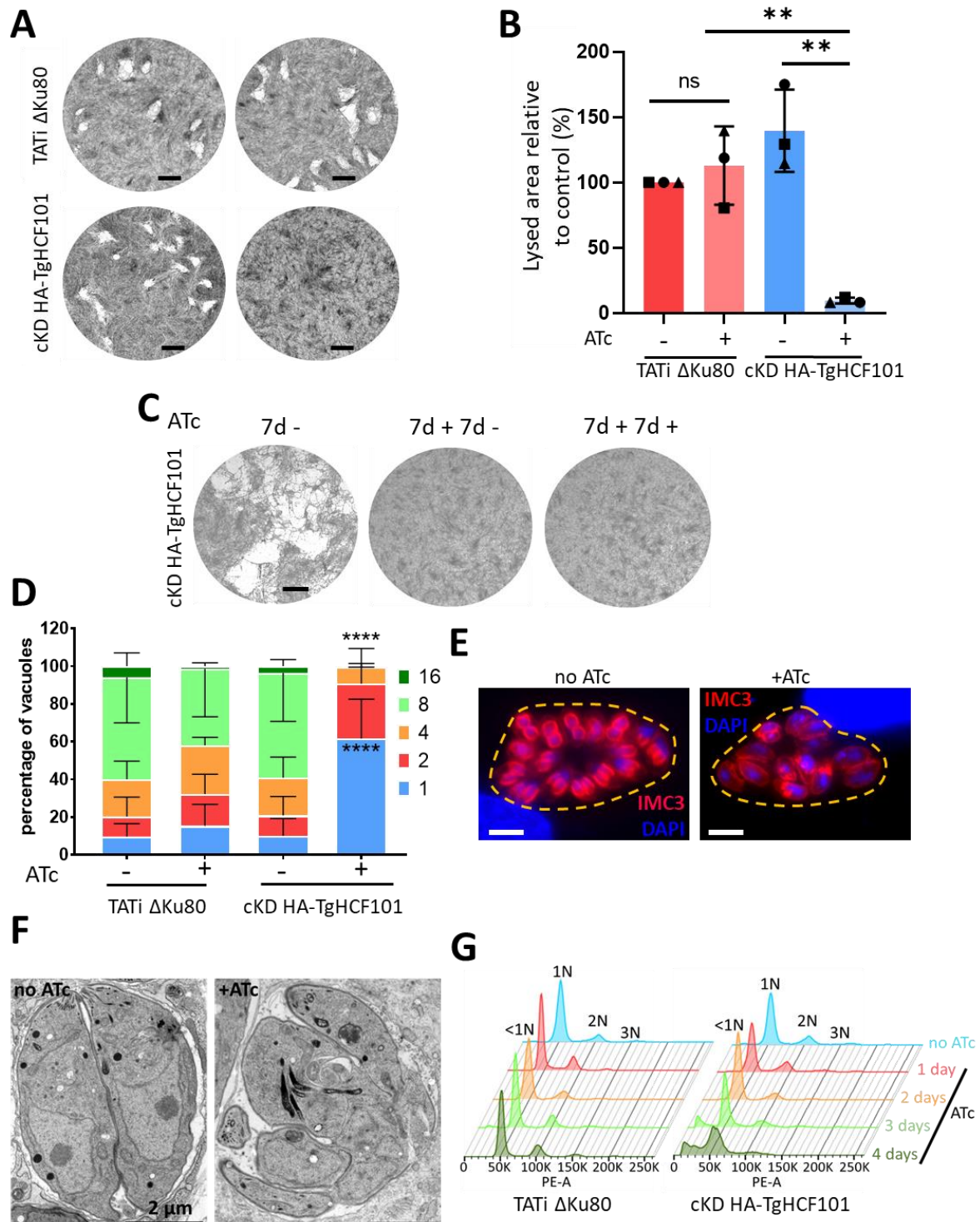
883

884 **Figure 1. TgHCF101 is a cytosolic protein.** A. Evolutionary relationship of proteins of the MRP family.  
 885 Eukaryotic sequences from HCF101 homologs were aligned and submitted phylogenetic analysis with

886 the unweighted paired group mean arithmetic method. Scale bar represents 0.4 residue substitution  
887 per site. NAR1 is used as an outgroup. The plant HCF101 homolog from *A. thaliana* is indicated by a  
888 green arrowhead, TgHCF101 by a yellow arrowhead and the two homologs present in *C. velia* are  
889 indicated by red arrowheads. **B.** Schematic representation of HCF101 homologs in *A. thaliana*  
890 (AtHCF101), in *T. gondii* (TGGT1\_318590) and in *C. velia* (Cvel\_23131, Cvel\_17212). Main domains are  
891 highlighted on the sequences; TP: Transit peptide, MIP18-like domain (or Domain of unknown function  
892 59, DUF59), the ATPase domain and DUF971. **C.** Strategy for generating the inducible knockdown of  
893 *TgHCF101* by promoter replacement and simultaneous N-terminal tagging of the TgHCF101 protein in  
894 the TATi  $\Delta$ Ku80 cell line. **D.** Diagnostic PCR for checking correct integration of using the primers  
895 mentioned in (B), on genomic DNAs of a transgenic parasite clone and of the parental strain. **E.**  
896 Immunoblot analysis of the cKD-TgHCF101 mutant and parental line showing efficient tagging and  
897 downregulation of TgHCF101 starting at 24h of treatment with ATc. Actin was used as a loading control.  
898 **F.** Immunofluorescence assay showing a cytosolic signal for TgHCF101 protein (labeled with an anti-  
899 HA), with no particular co-localization with the apicoplast (Apico, labeled with anti-PDH-E2), and total  
900 depletion of the protein after 48h of ATc treatment. DNA was stained with 4',6-diamidino-2-  
901 phenylindole (DAPI). Scale bar= 5  $\mu$ m.

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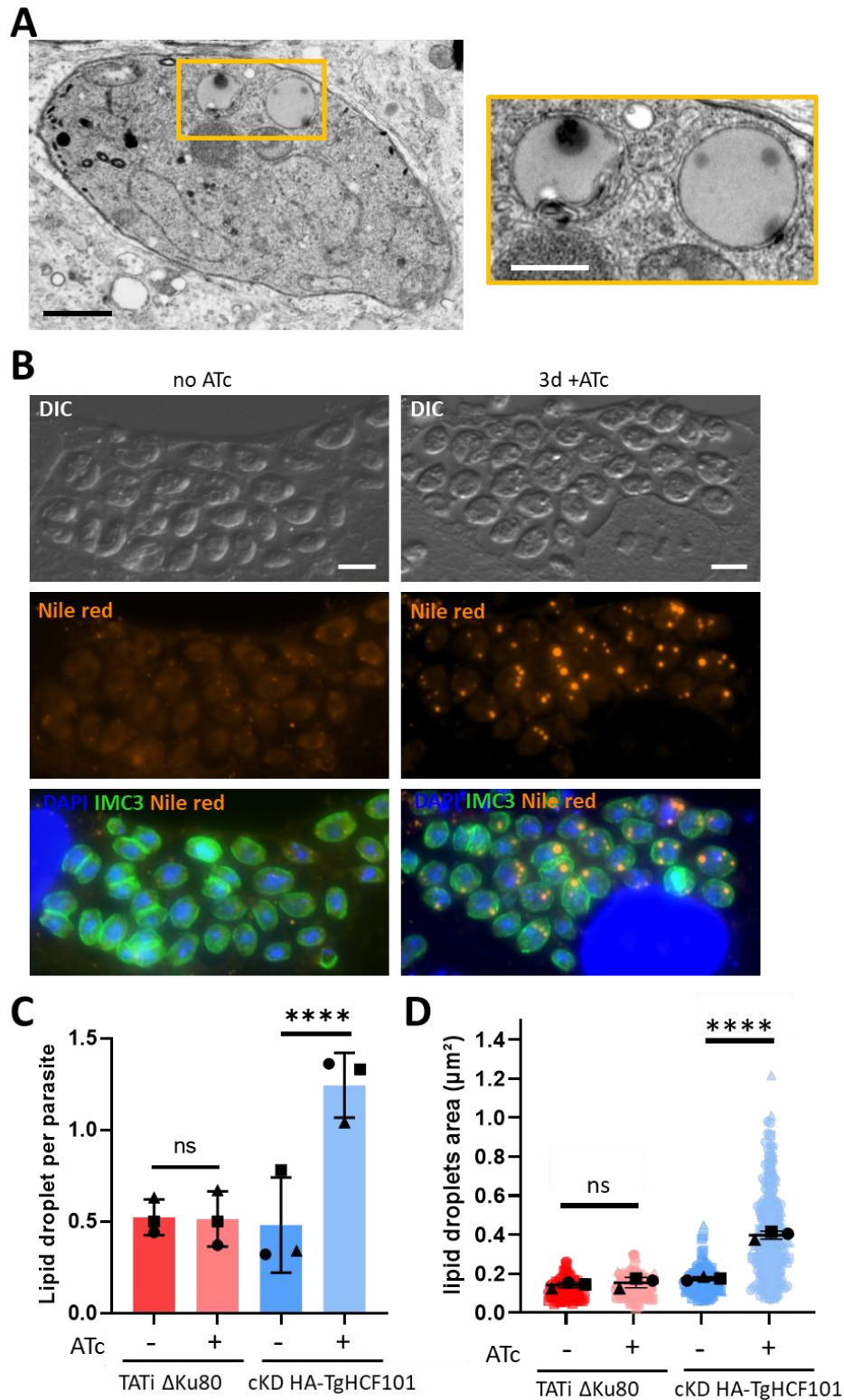
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904 **Figure 2. TgHCF101 is essential for parasite growth and survival.** **A.** Plaque assays were carried out by  
 905 infecting a monolayer of HFFs with TATi  $\Delta$ Ku80 or cKD-TgHCF101 cell lines for 7 days in the presence  
 906 or absence of ATc. Scale bar= 2 mm. **B.** Quantification of plaque area observed in (A). Results are  
 907 expressed as percentage of lysed area relative to control (TATi  $\Delta$ Ku80 -ATc, set as 100% for reference).  
 908 Values represented are mean  $\pm$  SD from n=3 independent biological replicates, \*\* p-value  $\leq$  0.01,  
 909 Student *t*-test **C.** Plaque assays were carried out with cKD-TgHCF101 parasites as described in (A) but  
 910 for the '7d+7d-' condition, ATc was washed-out after 7 days and parasites were allowed to grow for  
 911 another 7 days without ATc, while in the '7d+7d+' condition, ATc treatment was maintained for 7 more  
 912 days. The '7d -' control was kept without ATc for 7 days of growth. Scale bar= 2mm. **D.** Replication



913 assay of parental (TATi  $\Delta$ Ku80) and transgenic cell lines (cKD-TgHCF101): parasites were pre-cultured  
914 for 48h in the presence or absence of ATc and allowed to invade HFF-coated coverslips for another 24h  
915 in the presence or absence of ATc, for a total of up to 72h of treatment with ATc. Number of parasites  
916 per vacuole was quantified for each condition and expressed as a percentage, 200 vacuoles were  
917 counted for each condition. Values represented are mean  $\pm$  SD of 3 independent biological replicates,  
918 \*\*\*\* p-value  $\leq$  0.0001 by two-way ANOVA with Dunnett's multiple comparison test, showing a  
919 significant difference when comparing the TATi  $\Delta$ Ku80 control and cKD-TgHCF101+ATc parasites for  
920 percentage of vacuoles containing 1 or 8 parasites. **E.** DNA content analysis by flow cytometry on TATi  
921  $\Delta$ Ku80 and cKD-TgHCF101 parasites treated or not with ATc up to 4 days and stained with propidium  
922 iodide. 1N, 2N, 3N represents the ploidy with <1N correspond to parasite with less than 1 full nuclear  
923 DNA content. **F.** Immunofluorescence assay of cKD-TgHCF101 parasites showing asynchronous division  
924 of parasites growing in the same vacuole (outlined with a yellow dotted line) upon TgHCF101 depletion  
925 (+ATc condition: parasites pre-incubated for 48h with ATc and allowed to invade coverslips for another  
926 36h in the presence of ATc). Individual parasites and budding daughter cells are outlined by anti-IMC3  
927 antibody staining (magenta), DNA was stained with DAPI. Scale bar= 5  $\mu$ m. **G.** Electron microscopy of  
928 cKD-TgHCF101 parasites pre-incubated with ATc for 24h before being released from their host cell and  
929 allowed to reinvade for 24h in the presence (+ATc) or grown in absence of ATc (-ATc). Scale bar= 2  $\mu$ m.

930

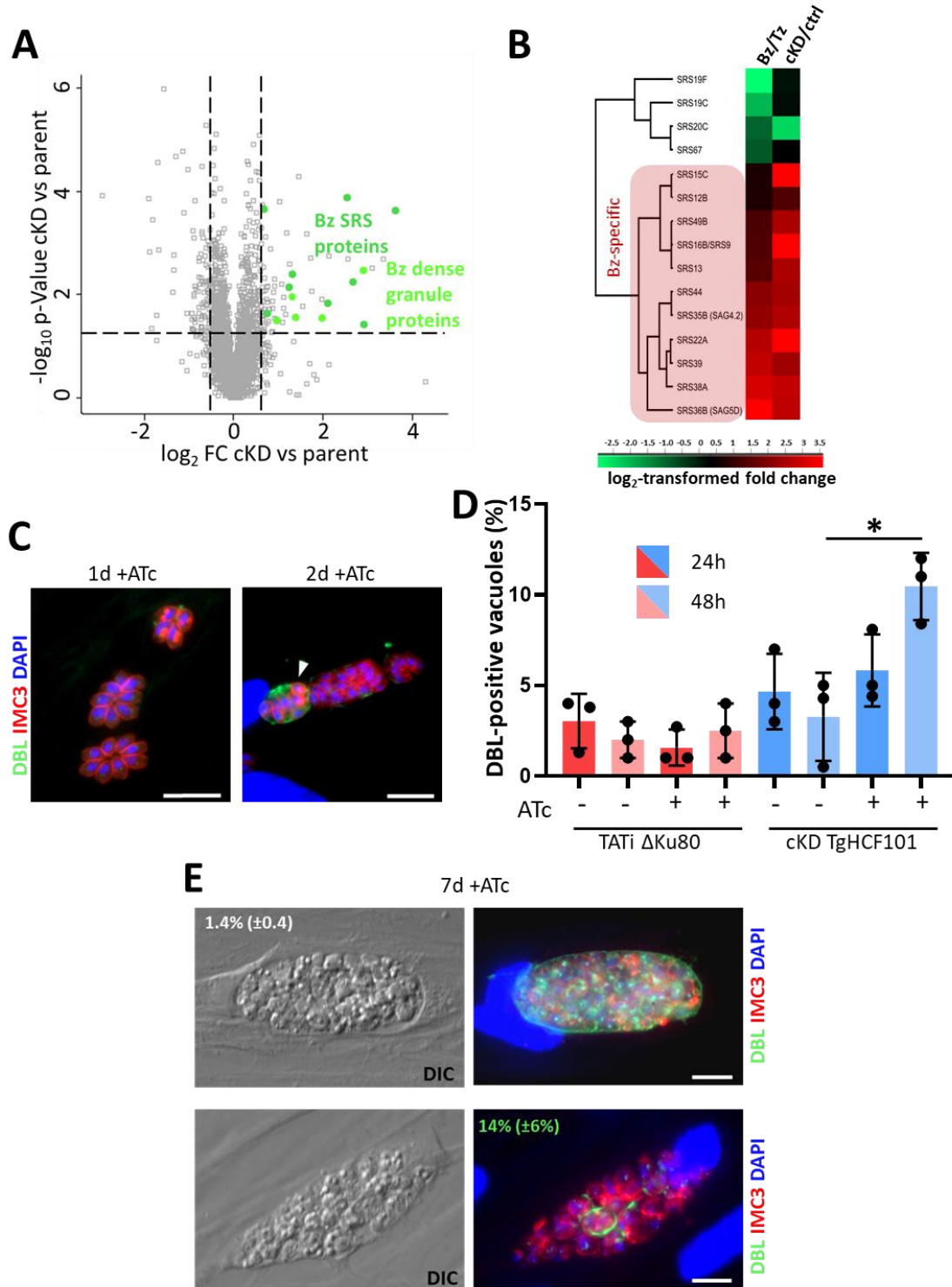


931

932 **Figure 3. TgHCF101 depletion induces lipid droplet accumulation.** A. Electron microscopy of cKD-  
 933 TgHCF101 parasites pre-incubated with ATc for 48h and allowed to reinvade for 24h in the presence  
 934 of ATc. Scale bar= 1  $\mu\text{m}$ . The panel on the right corresponds to a magnification of the selection on the  
 935 left panel, highlighting lipid droplets. Scale bar= 500 nm B. Fluorescent imaging of parasites from the  
 936 cKD-TgHCF101 cell line treated for 72h with ATc or in grown the absence of ATc, shows an  
 937 accumulation of lipid droplet upon TgHCF101 depletion. Lipid droplets were detected with Nile red

938 (orange), parasites are outlined with an anti-IMC3 antibody (green) and DNA is stained with DAPI. DIC=  
939 Differential interference contrast. Scale bar= 5  $\mu$ m. **C.** and **D.** correspond to the quantification of lipid  
940 droplet number and area, respectively. 100 parasites were analyzed per condition. the parental (TATi  
941  $\Delta$ Ku80) and transgenic (cKD-TgHCF101) parasites were grown in absence of ATc, or in presence of ATc  
942 for 72h. Values are represented as the mean  $\pm$  SD of n=3 independent biological replicates; ns, not  
943 significant (p-value>0.05), \*\*\*\* p-value  $\leq$  0.0001, Student's *t*-test.

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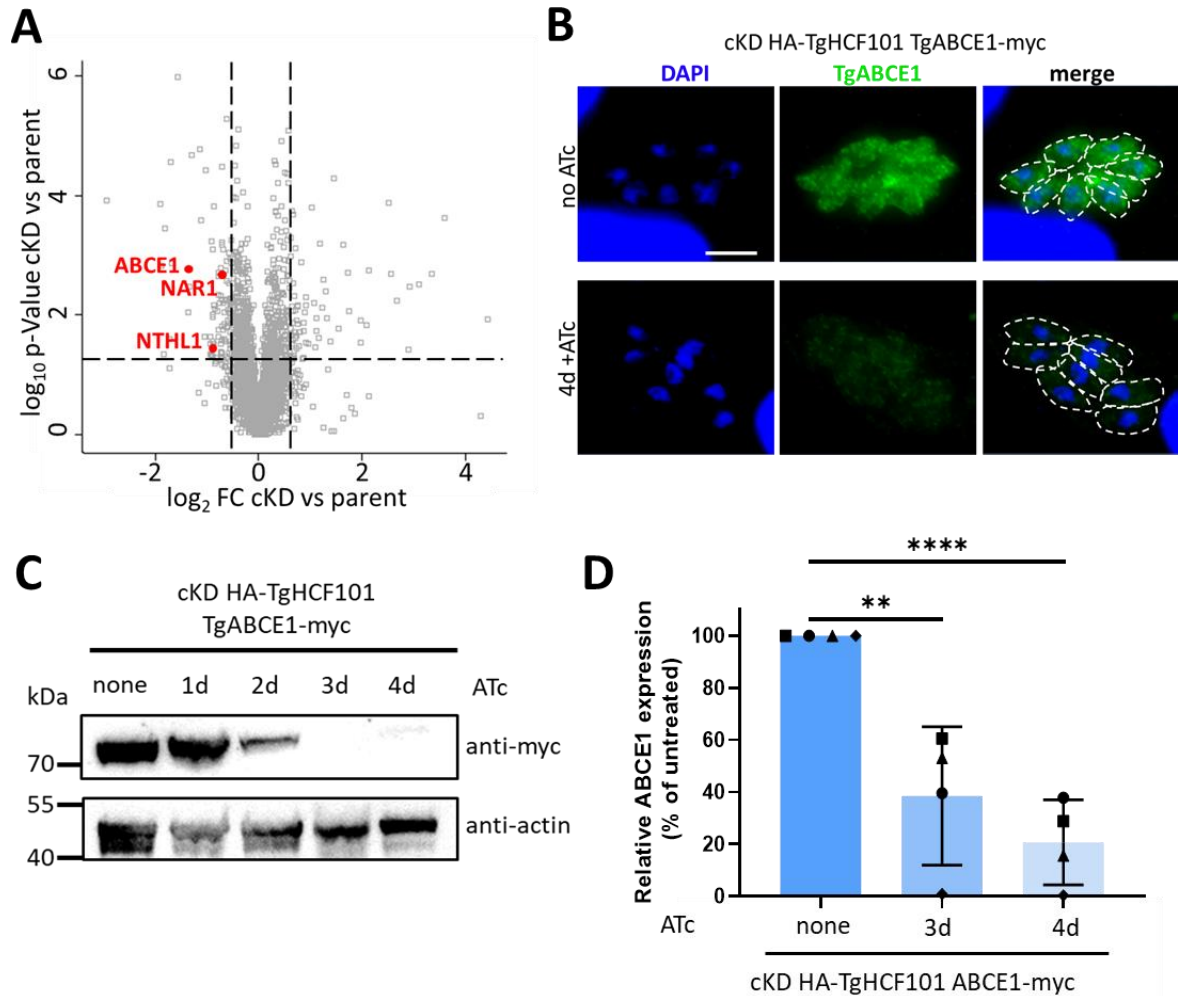


945

946 **Figure 4. TgHCF101-depleted parasites express bradyzoite-specific markers but are unable to**  
 947 **complete their conversion. A.** Volcano plot showing differential expression of proteins impacted by  
 948 TgHCF101 depletion after 72h of ATc treatment analyzed by label free quantitative proteomic. X-axis  
 949 corresponds to the  $\log_2$  of the fold-change (FC) and the Y-axis corresponds to the  $-\log_{10}$  of the p-value,  
 950 when comparing cKD-TgHCF101 expression values to the TATi  $\Delta$ Ku80 parental cell line. Statistical  
 951 analyses were performed with ANOVA on 4 independent biological replicates. Cut-offs were set at  
 952  $\leq 1.5$ - or  $\geq 1.5$ -FC and p-value  $\leq 0.05$ . Significant hits corresponding to stage-specific protein are  
 953 highlighted in green on the graph. **B.** Clustering of bradyzoite (Bz) or tachyzoite (Tz)-specific proteins

954 of the SRS family shows specific enrichment of bradyzoite proteins upon TgHCF101 depletion. **C.**  
955 Immunofluorescence assay of cKD-TgHCF101 treated for 24h or 48h with ATc, cyst wall is labelled with  
956 DBL, parasites periphery is outlined with anti-IMC3 antibody and DNA is stained with DAPI. Scale bar=  
957 10 $\mu$ m **D.** Corresponds to the quantification of the percentage of vacuoles presenting a DBL positive  
958 signal as shown in (C). Values are represented as the mean  $\pm$  SD of n=3 independent biological  
959 replicates, \* p-value  $\leq$  0.05, Student's *t*-test. **E.** Immunofluorescence assay of cKD-TgHCF101 treated  
960 for 7 days in the presence of ATc, cyst wall is labelled with DBL, parasites are outlined with anti-IMC3  
961 antibody and DNA is stained with DAPI. The percentage of DBL positive vacuoles of corresponding size  
962 (more than 2 parasites per vacuole, top; or 2 parasites per vacuole or less, bottom) is specified as mean  
963  $\pm$  SD of n=3 independent biological replicates. DIC= Differential interference contrast. Scale bar= 10  
964  $\mu$ m.

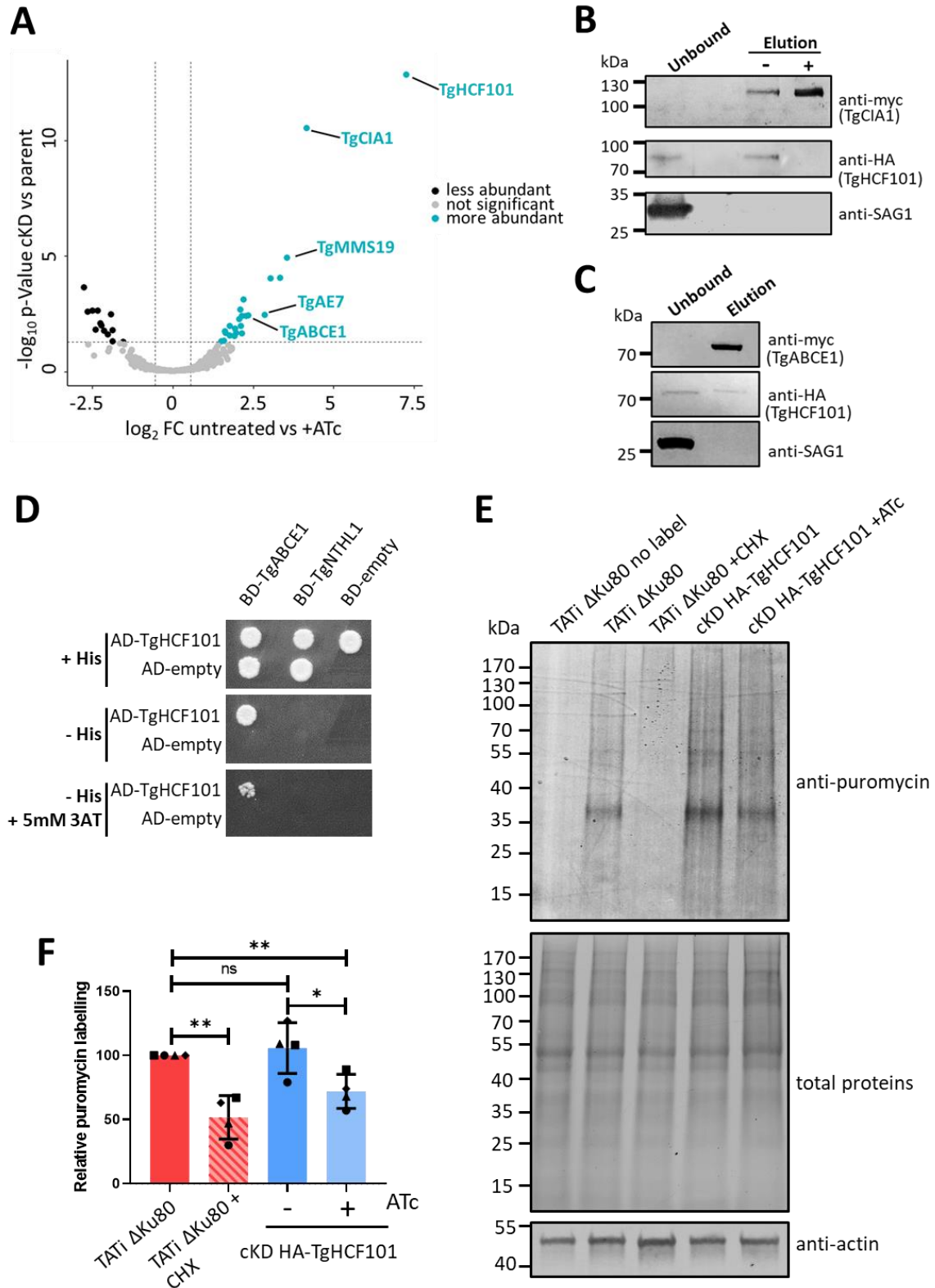




965

966 **Figure 5. TgHCF101 is involved in Fe-S cluster biogenesis of the CIA pathway.** **A.** Volcano plot showing  
 967 differential expression of proteins impacted by TgHCF101 depletion after 72h of ATc treatment  
 968 analyzed by label free quantitative proteomic. Cut-offs were set at  $\leq$ 1.5- or  $\geq$ 1.5-fold change (FC) and  
 969 p-value  $\leq$ 0.05. Significant hits corresponding to predicted Fe-S cluster proteins are highlighted in red  
 970 on the graph. **B.** Immunofluorescence assay for the detection of the myc-tagged protein TgABCE1 in  
 971 the cKD TgHCF101 genetic background. Parasites were pre-incubated for 48h in the presence of ATc  
 972 and allowed to invade HFF coated coverslips for another 48h in the presence of ATc. The control (no  
 973 ATc) was infected 24h prior to fixation. TgABCE1 was detected with anti-myc antibody and DNA was  
 974 stained with DAPI. Parasites periphery is outlined by white dotted lines. Scale bar=5  $\mu$ m. **C.** Immunoblot  
 975 analysis of TgABCE1 expression shows decrease upon TgHCF101 depletion up to 4 days of treatment  
 976 with ATc in the cKD HA-TgHCF101 TgABCE1-myc cell line. TgABCE1 expression was detected with anti-  
 977 myc antibody and anti-actin antibody was used as a loading control. **D.** Decrease of TgABCE1  
 978 expression upon TgHCF101 depletion was quantified by band densitometry analysis and normalized  
 979 on the loading control of each respective lane. The relative abundance of ABCE1 is presented as a  
 980 percentage relative to the untreated control, set as 100%, for each biological replicate. Values are  
 981 represented as the mean  $\pm$  SD from n=4 independent biological replicates, \*\* p-value  $\leq$  0.01, \*\*\*\* p-  
 982 value  $\leq$  0.0001, Student's *t*-test.

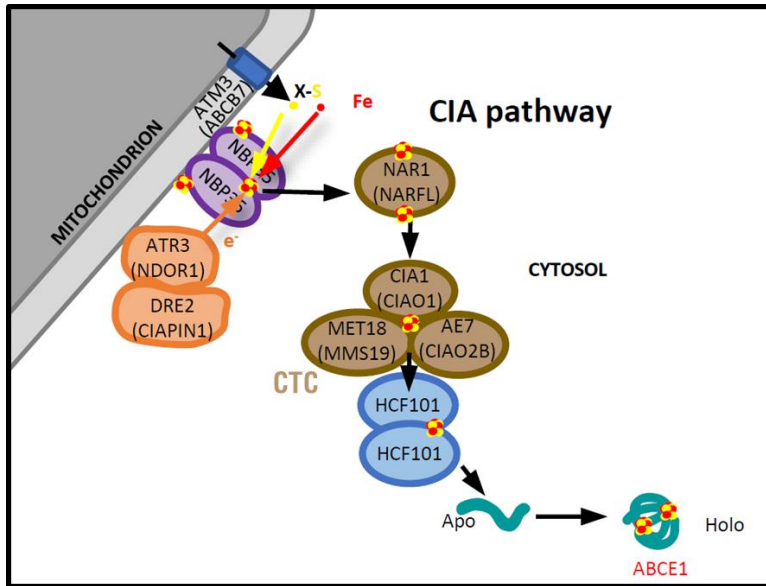
983



984

985 **Figure 6. TGHCF101 is associated to the CIA targeting complex and specifically interacts with**  
 986 **TgABCE1.** **A.** Volcano plot showing differential expression of TgHCF101 and co-immunoprecipitated  
 987 proteins in the cKD HA-TgHCF101 cell line after TgHCF101 depletion (with 72h of ATc treatment) or  
 988 not, as analyzed by quantitative proteomic. Cut-offs were set at  $\leq 1.5$ - or  $\geq 1.5$ -fold change and p-value  
 989  $\leq 0.05$ . Upregulated proteins are highlighted in blue, significant hits corresponding to predicted

990 proteins of the CIA targeting complex and target protein TgABCE1 were annotated on the graph. **B.**  
991 Immunoblot analysis of a reverse co-immunoprecipitation assay of the myc-tagged TgCIA1 protein in  
992 the cKD HA-TgHCF101 background shows specific co-immunoprecipitation of TgHCF101, which is  
993 absent upon depletion by ATc. The anti-SAG1 antibody was used as a control for unspecifically bound  
994 proteins. **C.** Immunoblot analysis of a reverse co-immunoprecipitation assay of the myc-tagged  
995 TgABCE1 protein showing TgHCF101 is co-immunoprecipitating. The anti-SAG1 antibody was used as  
996 a control for unspecifically bound proteins. **D.** TgHCF101 interacts with TgABCE1 in a Gal4-based yeast  
997 two-hybrid assay. Co-transformed YRG2 cells expressing AD- and BD-fusion proteins were plated on a  
998 control plate (+His, upper panel) for checking cell fitness and on the Y2H test plate (-His, mid panel),  
999 and plates were incubated at 30°C. Yeast growth was recorder after 5 days. At the cell concentration  
1000 used ( $OD_{600} = 0.05$ ), the observed interaction between AD-TgHCF101 and BD-TgABCE1 fusion proteins  
1001 was strong enough to allow cell growth in the presence of 5 mM 3AT inhibitor (-his + 3AT lower panel).  
1002 None of the proteins tested alone exhibited *HIS3* transactivation capacities. Results shown here are  
1003 representative of three independent experiments. **E.** Immunoblot analysis of puromycin incorporation  
1004 in the parental (TATi  $\Delta$ Ku80) and cKD TgHCF101 cell lines untreated or treated with ATc for 72h. TATi  
1005  $\Delta$ Ku80 treated with cycloheximide (CHX) was used as a control for translation inhibition. The  
1006 puromycin signal was detected with anti-puromycin antibody, and total protein content was visualized  
1007 by stain-Free imaging technology. Anti-actin antibody was also used as a loading control. **F.** Variation  
1008 of puromycin incorporation in the different conditions was quantified by band densitometry and  
1009 normalized on the total protein content of each respective lane. Puromycin labelling is presented as a  
1010 percentage relative to the untreated control, set as 100% for each biological replicate. Values are  
1011 represented as the mean and SD of 4 independent biological replicates; \* p-value  $\leq 0.05$ ; \*\* p-value  $\leq$   
1012 0.01; ns, not significant (p-value  $\geq 0.05$ ), Student's *t*-test.  
1013



1014

1015 **Figure 7. Schematic representation of the putative organization of the CIA pathway in *T. gondii*.** This  
1016 scheme places TgHCF101 as a Fe-S transfer protein from the CIA transfer complex (CTC) to client  
1017 protein ABCE1. Plant nomenclature was used for the purpose of the figure, but names for human  
1018 homologs are mentioned between brackets when appropriate.

1019

1020 **Supplemental table legends**

1021 **Table S1.** Sequences used for the phylogenetic analysis displayed in Fig. 1A.

1022 **Table S2.** Proteins with higher expression upon depletion of TgHCF101 as found by label-free  
1023 quantitative proteomics. For each protein candidate (with [www.ToxoDB.org](http://www.ToxoDB.org) and [www.Uniprot.org](http://www.Uniprot.org)  
1024 identifier),  $\log_2$  of the different ratio were calculated between the mean MaxQuant LFQ values found  
1025 for the cKD HA-TgHCF101 mutant and the TATi  $\Delta$ Ku80 parental cell line.  $-\log_{10}(\text{pvalue})$  is also provided.  
1026 Putative subcellular localization was obtained from the hyperLOPIT data available on ToxoDB.org, or  
1027 by manual annotation. CRISPR fitness score and transcriptomic data for tachyzoites (Tz) and  
1028 bradyzoites (Bz) were obtained from ToxoDB.org.

1029 **Table S3.** Proteins with lower expression upon depletion of TgHCF101 as found by label-free  
1030 quantitative proteomics. For each protein candidate (with [www.ToxoDB.org](http://www.ToxoDB.org) and [www.Uniprot.org](http://www.Uniprot.org)  
1031 identifier),  $\log_2$  of the different ratio were calculated between the mean MaxQuant LFQ values found  
1032 for the cKD HA-TgHCF101 mutant and the TATi  $\Delta$ Ku80 parental cell line.  $-\log_{10}(\text{pvalue})$  is also provided.  
1033 Putative subcellular localization was obtained from the hyperLOPIT data available on ToxoDB.org, or  
1034 by manual annotation. CRISPR fitness score and transcriptomic data for tachyzoites (Tz) and  
1035 bradyzoites (Bz) were obtained from ToxoDB.org.

1036 **Table S4.** Proteins identified as co-immunoprecipitating with TgHCF101 through comparative mass  
1037 spectrometry analysis of immunoprecipitated extracts form the cKD HA-TgHCF101 cell line frown or  
1038 not in the presence of ATc.

1039 **Table S5.** Oligonucleotides used in this study.