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The apicoplast is important for the viability and persistence of *Toxoplasma gondii* bradyzoites

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Toxoplasma gondii is responsible for toxoplasmosis, a disease that can be serious when contracted during pregnancy, but can also be a threat for immunocompromised individuals. Acute infection is associated with the tachyzoite form that spreads rapidly within the host. However, under stress conditions, some parasites can differentiate into cyst-forming bradyzoites, residing mainly in the central nervous system, retina and muscle. Because this latent form of the parasite is resistant to all currently available treatments, and is central to persistence and transmission of the parasite, specific therapeutic strategies targeting this developmental stage need to be found. _T. gondii_ contains a plastid of endosymbiotic origin called the apicoplast, which is an appealing drug target because it is essential for tachyzoite viability and contains several key metabolic pathways that are largely absent from the mammalian host. Its function in bradyzoites, however, is unknown. Our objective was thus to study the contribution of the apicoplast to the viability and persistence of bradyzoites during chronic toxoplasmosis. We have used complementary strategies based on stage-specific promoters to generate conditional bradyzoite mutants of essential apicoplast genes. Our results show that specifically targeting the apicoplast in both in vitro or in vivo-differentiated bradyzoites leads to a loss of long-term bradyzoite viability, highlighting the importance of this organelle for this developmental stage. This validates the apicoplast as a potential area to look for therapeutic targets in bradyzoites, with the aim to interfere with this currently incurable parasite stage.

Toxoplasma gondii | chronic toxoplasmosis | plastid | stage differentiation

Toxoplasma gondii is an intracellular parasitic protist responsible for toxoplasmosis, a ubiquitous disease affecting humans, which can lead to severe forms during pregnancy or in immunosuppressed individuals (1). Acute toxoplasmosis is associated with the tachyzoite form of these parasites, which spreads rapidly within the host and has the ability to cross physiological barriers, but is generally controlled by the immune system in immunocompetent hosts (2). The genus *Toxoplasma* contains only one species, but there are many different strains, which are grouped according to their virulence and pathogenicity in mouse models (3). The pathogenic outcome is closely linked to the ability of the parasite to convert from fast-replicating tachyzoites to persisting encysted stages that can be found predominantly in neurons and muscle tissues and are called bradyzoites (4). These developmental forms of the parasite, which are the hallmark of chronic infection, are supposedly less active metabolically and slower to replicate than tachyzoites (5). However, they play a crucial role in *T. gondii* transmission, as well as in immune escape and survival of the parasites under stressful conditions (6).

The process of stage conversion is a continuum lasting for several days that leads to marked differences between tachyzoites and bradyzoites. For instance the membrane of the parasitophorous vacuole (PV, the compartment in which the parasite replicates) transforms into a heavily glycosylated cyst wall (7, 8). Also, several aspects of parasite metabolism change drastically, as illustrated by the accumulation of amylopectin granules in bradyzoites (9) and the expression of different stage-specific isoforms of metabolic enzymes (10–14). Thus unsurprisingly, stage conversion is accompanied by a considerable change in protein expression driven by stage-specific promoters. Transcriptomic analyses of in vivo-generated cysts have for instance revealed that the expression levels of hundreds of genes are potentially changing between bradyzoites and tachyzoites (15, 16). The specific metabolic requirements of bradyzoites are so far poorly characterized, largely due to the technical challenges associated with studying this developmental stage: although in vitro differentiation can be achieved by applying stresses to a cystogenic *T. gondii* strain or by infecting specific host cell types, in vitro-derived bradyzoites are difficult to keep in long-term culture and they usually do not reach the stage of complete maturity that is observed in mouse brain–derived bradyzoites (17–20).

Significance

In its intermediate hosts, the parasite *Toxoplasma gondii* can persist as a cyst-contained developmental form that might reactivate and cause severe pathologies. Importantly, this form is resistant to current antiparasitic drugs. *T. gondii* harbors a plastid of endosymbiotic origin called the apicoplast, containing important and potentially druggable metabolic pathways, but whose contribution to the fitness and viability of persistent parasites has never been assessed. We have generated conditional mutants specifically affected for the homeostasis of the apicoplast in cyst-contained parasites and showed that this organelle is important for persistence of these particular developmental forms. Our work thus validates the apicoplast as a relevant drug target in the context of chronic *T. gondii* infection.

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As intracellular developmental stages, both PV-contained tachyzoites and cyst-enclosed bradyzoites are tightly interconnected metabolically with their host cells to ensure optimal proliferation or persistence (21). While *T. gondii* relies on specific host cell resources for survival, it is also able to synthesize de novo a number of important metabolites. One key parasite organelle for metabolite production is the apicoplast. This plastid of secondary endosymbiotic origin has lost its photosynthetic capacity (22), but it nevertheless harbors four main metabolic pathways, which have all been shown to be essential for the in vitro fitness of *T. gondii* tachyzoites (23, 24). These are important for the synthesis of fatty acids (FAs, via the FASII pathway), heme (together with the mitochondrion), isoprenoid precursors and iron–sulfur clusters. Because of the prokaryotic origin and the metabolic importance of most of the apicoplast-hosted pathways, this organelle is particularly attractive as a potential drug target and compounds inhibiting translation in the apicoplast, like clindamycin, are already used against acute toxoplasmosis (25).

However, to this day there is no drug available to clear the cyst form that persists during the chronic phase of infection, and this has major implications for pathogenesis. Clearly, curing patients from the infection is of paramount importance to prevent parasite reactivation. Several compounds, including atovaquone and quinolone derivatives, or even combinatorial strategies involving apicoplast-targeting drugs like clindamycin and spiramycin, are able to limit cyst burden in animal models of chronic toxoplasmosis (26–32). However, most fail to completely eradicate bradyzoites, and some are even known to be potent inducers of stage conversion in vitro (33, 34). Yet, it is still unclear whether the resistance to these drugs is due to poor accessibility of brain-localized cysts or to the metabolically quiescent nature of the bradyzoites.

The metabolic contribution of the apicoplast to the viability and persistence of these developmental stages is still a major conundrum. Although Atg8, a protein that is important for maintaining apicoplast homeostasis (35) was found to be essential for bradyzoite viability, its involvement in canonical autophagy, which is also essential for the survival of this particular stage, did not allow demonstrating the essentiality of the organelle (36). To get insights into the importance of the organelle in the context of chronic *T. gondii* infection, we have thus generated conditional mutants more specifically affected in apicoplast function at the bradyzoite stage. As the apicoplast is essential to the viability of tachyzoites, which are a necessary step prior to obtaining bradyzoites, we needed to adopt a conditional strategy for targeting the organelle. Most conditional expression systems available for *T. gondii* involve the use of small molecules (37); however, the bioavailability of these compounds may be difficult to regulate in the in vivo mouse model and most of them may not efficiently cross the blood–brain barrier to reach the tissue cysts. We thus genetically engineered parasite strains to impact apicoplast functions specifically in bradyzoites and without the use of external compounds, by using stage-specific promoters. We generated mutants either affected for apicoplast-related metabolism or in the actual structure and division process of the organelle. Through in vitro and in vivo phenotypic analyses, we showed that in both cases the viability or development of bradyzoites was impaired. Our study thus validates the apicoplast as a potential drug target in the context of chronic toxoplasmosis.

Results

Specific Downregulation of the Apicoplast Triosephosphate Translocator 1 (APT1) at the Bradyzoite Stage. To generate a parasite line impacted for apicoplast homeostasis, we first targeted the APT1. APT1 is involved in the import of phosphoenol pyruvate and triose phosphates, which are important precursors for the FASII system and for isoprenoid synthesis in the organelle (Fig. 1*A*), and as such it is essential to the survival of *T. gondii* tachyzoites (38). Although the metabolic requirements of the bradyzoites are not completely elucidated yet, we reasoned that depleting a transporter which is needed for two of the most important apicoplast-hosted metabolic pathways would be likely to have an impact. To generate a conditional mutant specifically affected in bradyzoites, we used CRISPR/Cas9 to edit the endogenous *APT1* locus in the cystogenic Prugniaud (Pru) strain: we inserted a cassette coding for a GFP-fused version of the APT1 protein driven by a tachyzoite stage–specific *SAG1* promoter (Fig. 1*B*). Proper integration in the resulting cell line, named pSAG1-APT1, was verified by PCR (*SI Appendix*, Fig. S1*A*). Using costaining with TgAtrx1, a marker of the periphery of the apicoplast (39), we also checked that the promoter change and the addition of the GFP tag were not affecting the localization of APT1 at the organelle (*SI Appendix*, Fig. S1*B*). However, it should be noted that the *SAG1* promoter is potentially a much stronger promoter than the native *APT1* promoter according to comparative analysis of RNA expression level (6). Accordingly, we performed semiquantitative RT-PCR analyses on tachyzoites that confirmed at least a threefold increase in expression for the *APT1* mRNA upon promoter change (*SI Appendix*, Fig. S1 *C* and *D*).

Conversion of tachyzoites into bradyzoites can be initiated in vitro by applying a variety of stresses like alkaline pH, heat shock, nutrient starvation, or the use of specific drugs (4). For instance, alkaline pH stress–induced differentiation, which is performed in absence of CO_2, is commonly used to induce conversion to bradyzoites with minimal toxicity to host cells. We thus used this stress on the pSAG1-APT1 cell line and performed immunoblot analysis that showed a decrease in expression of the *SAG1* promoter–driven APT1 protein in in vitro-generated bradyzoites (Fig. 1*C*). We performed fluorescence microscopy on differentiating parasites using a lectin from the plant *Dolichos biflorus* (DBL) that recognizes the SRS44/CST1 cyst wall glycoprotein that accumulates during differentiation (40). Strikingly, pSAG1-APT1 expression was tightly coordinated with SAG1 expression, and consequently APT1 was found to be largely absent from more mature cysts (Fig. 1 *D* and *E*). Quantification showed that the proportion of cysts displaying partial or complete loss of the pSAG1-APT1 signal increased over time during pH stress–induced differentiation (Fig. 1*F*).

Overall, our data show that the *SAG1* promoter replacement strategy leads to an efficient downregulation of APT1 upon in vitro differentiation of bradyzoites.

Downregulation of APT1 Leads to Perturbation of Apicoplast Homeostasis and Impacts the Development and Viability of In Vitro-Generated Bradyzoites. We next evaluated the impact of APT1 downregulation on the overall homeostasis of the apicoplast. Depleting APT1 in tachyzoites has been shown not to have an immediate impact on the morphology of the organelle (38). However, this translocator is important for isoprenoid production, whose perturbation leads to loss of the organelle in the related malaria-causing *Plasmodium* parasites (41, 42), so we anticipated that long-term depletion could also have an effect in bradyzoites. Costaining of APT1 with TgCpn60 [a marker of the apicoplast lumen (43)] during the differentiation process showed a disappearance of both proteins in more mature cysts (Fig. 2*A*). Quantification showed that only about 35% of the cysts still contained an intact TgCpn60 signal after 2 wk of pH

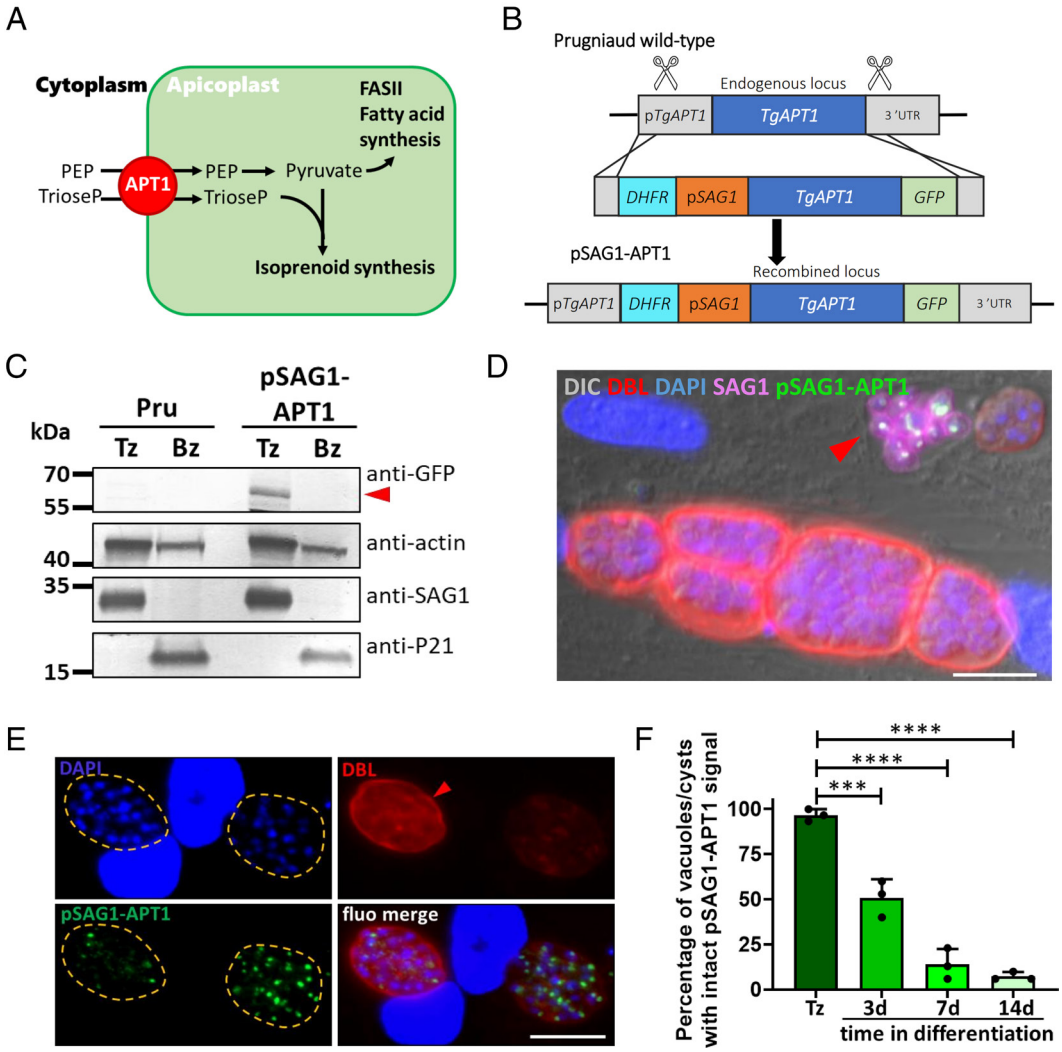

Fig. 1. Conditional depletion of APT1 at the bradyzoite stage. (*A*) Schematic representation of APT1 function at the apicoplast and its implication upstream of two important metabolic pathways. (*B*) Scheme describing the strategy to edit the *APT1* locus by homologous recombination, to express a copy of the *APT1* gene under the dependence of a tachyzoite-specific *SAG1* promoter and allowing fusion of APT1 with a C-terminal GFP. (*C*) Immunoblot analysis of protein extracts from tachyzoites (Tz) and 14 d in vitro-differentiated (alkaline stress) bradyzoites (Bz) with an anti-GFP antibody to detect the expression of APT1-GFP (arrowhead) under the dependence of the *SAG1* promoter. Actin was used as a loading control, SAG1 and P21 as tachyzoite and bradyzoite markers, respectively. (*D*) Microscopic imaging of pSAG1-APT1 parasites submitted to alkaline pH stress for 7 d shows that APT1 expression (green) is coordinated with the expression of SAG1 (magenta) in nondifferentiated parasites (arrowhead). Cyst walls were labeled with *D. biflorus* lectin (DBL). (Scale bar, 10 µm.) (*E*) Microscopic imaging of pSAG1-APT1 parasites differentiated as in *D*. Cyst walls were labeled with DBL or outlined with dashed lines. A more intense DBL labeling outlines a more mature cyst (arrowhead). DNA was labeled with DAPI. (Scale bar, 10 µm.) (*F*) The GFP-APT1 signal in vacuoles containing tachyzoites, or cysts generated by alkaline pH–induced differentiation, was quantified and shows progressive signal loss upon stage conversion. Data are mean from $n = 3$ independent experiments +SD. ***$P \leq 0.001$, ****$P \leq 0.0001$, by one-way ANOVA followed by Tukey post hoc test.

stress–induced differentiation (Fig. 2*B*). As this could reflect a specific impact on this protein marker rather than a more general organelle defect, we also stained for another apicoplast protein, the E2 subunit of the pyruvate dehydrogenase (44) and observed a similar loss (*SI Appendix*, Fig. S2). In cysts presenting defaults, both apicoplast markers were extensively lost (*SI Appendix*, Figs. S2 and S3). Overall, this suggests that APT1 loss subsequently leads to a perturbation of several apicoplast proteins, if not the whole organelle.

To assess the impact of APT1 downregulation on in vitro cyst development, we imaged cysts after 2 wk of differentiation and then measured cyst area (Fig. 2*C*). We found the mean area to be significantly lower in the pSAG1–APT1 cell line compared with the parental Pru cell line. This suggests that APT1 depletion upon in vitro differentiation has a negative impact on cyst development. We next sought to assess the impact of APT1 depletion on bradyzoite viability using plaque assay as a proxy, as described previously (36). Cysts were generated in vitro, bradyzoites were released by pepsin treatment and APT1-negative parasites were isolated with a cell sorter based on the absence of a GFP signal, and seeded on host cell monolayers (*SI Appendix*, Fig. S4 *A* and *B*). APT1-negative bradyzoites were found to generate many fewer and much smaller plaques than the Pru parental cell line (Fig. 2 *D*–*F*). Of note, pSAG1-APT1 tachyzoites did not show any particular fitness defect compared with the Pru parental cell line by plaque assay. (Fig. 2*D* and *SI Appendix*, Fig. S5 *A* and *B*).

In conclusion, our results indicate that specific depletion of APT1 in in vitro-generated bradyzoites leads to visible consequences on the homeostasis of the apicoplast and on bradyzoite viability.

pSAG1–APT1 Parasites Induce a Severe Acute Phase in Mice, yet Ex Vivo-Generated Bradyzoites Are Impaired in Fitness. While in vitro-derived cysts are certainly a useful model, some important

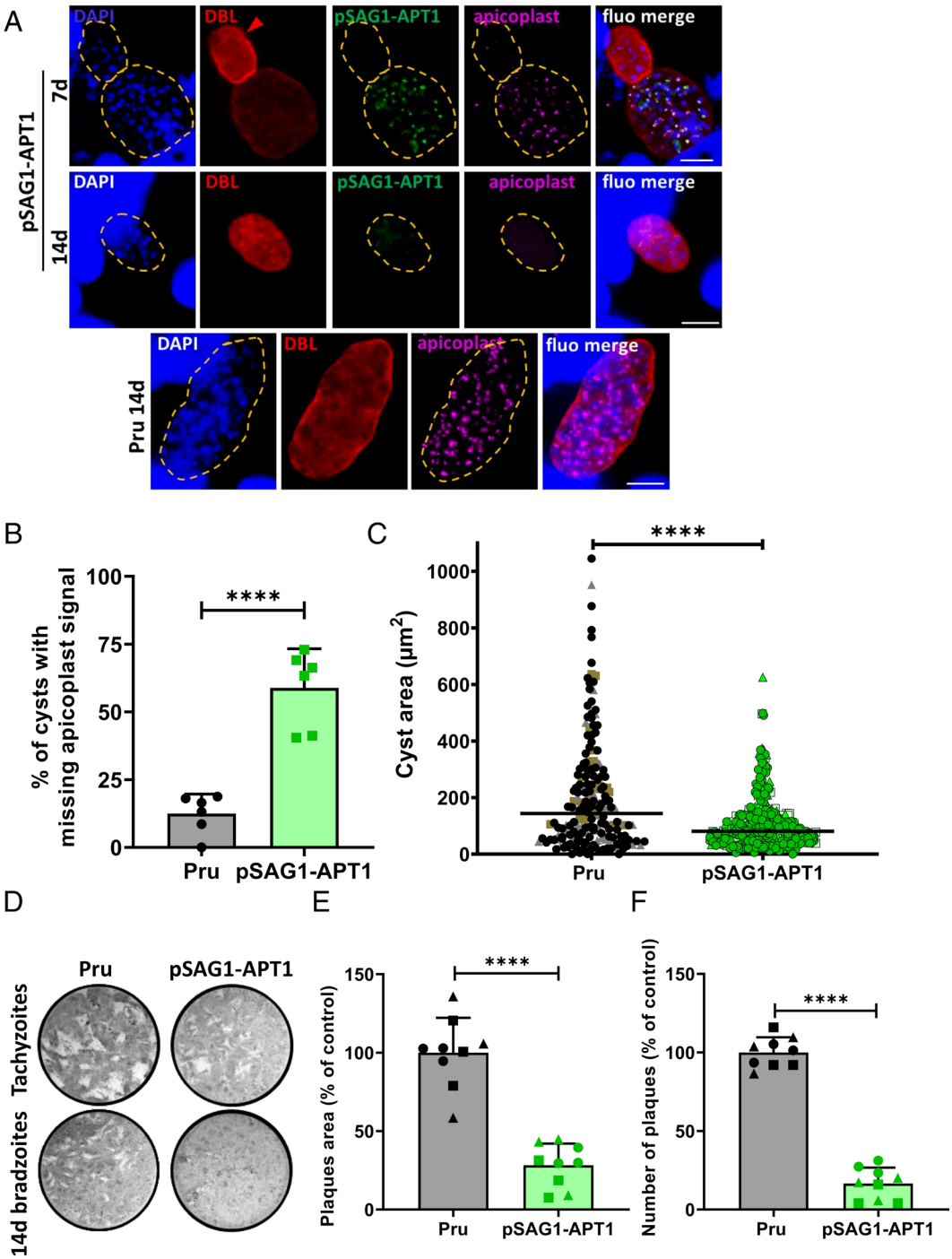

Fig. 2. Depletion of APT1 during in vitro differentiation impacts apicoplast maintenance and parasite development and viability. (*A*) Alkaline pH–induced in vitro differentiation of pSAG1-APT1 parasites for 7 or 14 d leads to the disappearance of APT1 and of the Cpn60 apicoplast marker (magenta) in mature cysts that are more intensely labeled with DBL (arrowhead). A 14-d-differentiated cyst of the Pru cell line is shown as a control. Cyst wall is outlined with dashed lines. DNA was stained with DAPI. (Scale bar, 10 µm.) (*B*) The integrity of the Cpn60-labeled apicoplast signal was quantified after 14 d of alkaline pH–induced differentiation and showed a significant loss in the pSAG1-APT1 cell line. Data are mean from $n = 6$ independent experiments +SD. ****$P \leq 0.0001$, unpaired Student's t test. (*C*) Quantification of the area of in vitro-differentiated cysts kept for 14 d under alkaline pH stress shows that the pSAG1-APT1 cell line generates smaller cysts. Data are mean from $n = 3$ independent experiments. Symbols are matched between identical experimental groups. ****$P \leq 0.0001$, nonparametric Mann–Whitney test. (*D*) Plaque assays were carried out by infecting HFF monolayers with tachyzoites or bradyzoites obtained after 14 d of alkaline pH stress and flow cytometry-based isolation. GFP-negative bradyzoites of the pSAG1-APT1 cell line generates less and smaller plaques than the Pru control cell lines. Measurements of lysis plaque area (*E*) and plaque number (*F*) highlight a significant defect in the lytic cycle of pSAG1-APT1 bradyzoites. Values are means of nine values (representing $n = 3$ independent experiments, containing three technical triplicates) +SD. Symbols are matched between identical experimental groups. Mean value for the Pru cell line was set to 100% as a reference. ****$P \leq 0.0001$, unpaired Student's t test.

differences have been observed with in vivo-derived cysts (20), thus we sought to confirm our findings with additional assays in an animal model. To this end, we infected CBA mice with the pSAG1-APT1 and Pru control cell lines. In three independent experiments we observed an excessive mortality and a more pronounced body mass loss in the surviving animals for mice infected with pSAG1-APT1 tachyzoites (Fig. 3 *A* and *B*), hinting that infection with this mutant leads to a severe acute phase. As dendritic cells act

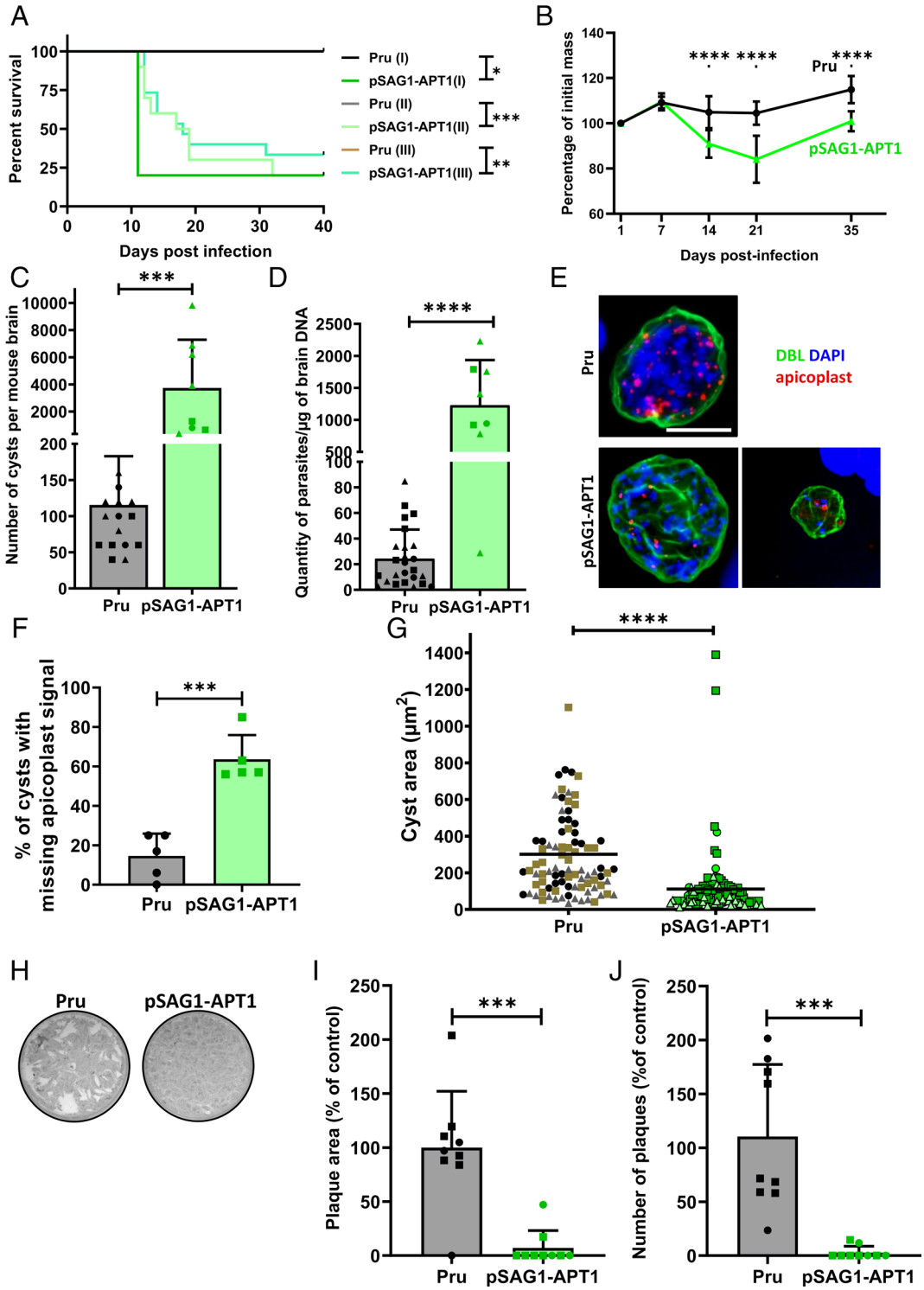

Fig. 3. Infection of mice with pSAG1-APT1 parasites leads to a severe acute phase and high cyst burden but generates essentially nonviable bradyzoites. (*A*) Survival curves of CBA mice infected with 100 parasites of the Pru or pSAG1-APT1 cell lines shows that the latter induces an excessive mortality during acute phase. Data from $n = 3$ independent experiments are plotted. $^{*}P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, log-rank Mantel-Cox test. (*B*) Monitoring of body mass of mice infected with Pru or pSAG1-APT1 tachyzoites. Infection with pSAG1-APT1 induces a more important mass loss than the control cell line, reflecting a more severe acute phase. Data represent mean from $n = 3$ independent experiments +SD. $^{****}P \le 0.0001$, unpaired Student's *t* test. (*C*) Quantification of cyst burden in infected mice 40 d postinfection. Values are from $n = 3$ independent experiments and are expressed as means +SD. Symbols are matched between identical experimental groups. $^{***}P \le 0.001$, unpaired Student's *t* test. (*D*) Parasite burden measured by qPCR on genomic DNA from infected mice. Values are from $n = 3$ independent experiments and are expressed as means +SD. Symbols are matched between identical experimental groups. $^{****}P \le 0.0001$, unpaired Student's *t* test. (*E*) Labeling of mouse brain–derived cysts with anti-Cpn60 shows partial loss of the apicoplast in the pSAG1-APT1 cell line. (Scale bar, 10 µm.) (*F*) Quantification of Cpn60 signal in brain-derived pSAG1-APT1 cysts shows that they display partial or total signal loss 40 d postinfection. Shown are the mean +SD from $n = 5$ independent experiments, at least 12 cysts for each condition were assessed. $^{***}P \le 0.01$, unpaired Student's *t* test. (*G*) Quantification of the area of brain-derived cysts obtained 40 d postinfection shows that the pSAG1-APT1 cell line generates smaller cysts. Data are mean from $n = 3$ independent experiments. Symbols are matched between identical experimental groups. $^{****}P \le 0.0001$, nonparametric Mann–Whitney test. (*H*) A representative plaque assay carried out by infecting HFF monolayers with brain-derived bradyzoites obtained after 40 d of infection shows that the pSAG1-APT1 cell line generates very few plaques. Measurements of lysis plaque area (*I*) and plaque number (*J*) confirms that there is a significant defect in the lytic cycle of brain-derived pSAG1-APT1 bradyzoites. Values are means from nine replicates from $n = 2$ independent experiments +SD. Mean value for the Pru cell line was set to 100% as a reference. Symbols are matched between identical experimental groups. $^{***}P \le 0.001$, unpaired Student's *t* test.

early both as immune response mediators and as parasite carriers that facilitate the dissemination of the infection and may play a key role in the acute phase (45), we wanted to evaluate the ability of pSAG1-APT1 tachyzoites to infect these cells in vitro using a cytometry-based assay (*SI Appendix*, Fig. S5 *C* and *D*) (46). Yet, our results showed that pSAG1-APT1 tachyzoites do not infect dendritic cells more efficiently than the Pru cell line (*SI Appendix*, Fig. S5*E*).

Tissue cysts were recovered from the brains of infected mice at 40 d postinfection and quantified by microscopic observation after DBL staining and parasite load was also quantified by qPCR (Fig. 3 *C* and *D*). Mice infected with the pSAG1-APT1 cell line had a much higher cyst burden than the Pru parental cell line, which could either be related to the more severe acute phase observed with these parasites, or illustrate more frequent parasite reactivation. Tissue cysts were imaged to detect the presence of an apicoplast, and a majority of pSAG1-APT1 cysts harbored bradyzoites missing apicoplast-resident proteins (Fig. 3 *E* and *F* and *SI Appendix*, Fig. S2 *D* and *E*). Using spectral flow cytometry to reduce the autofluorescence background we initially observed in in vitro-differentiated bradyzoites (*SI Appendix*, Fig. S4 *A* and *B*), we could see that very few APT1-GFP-positive bradyzoites remain after in vitro or in vivo differentiation, demonstrating an efficient downregulation of APT1 (*SI Appendix*, Fig. S4 *C–E*). We also found that the size of pSAG1-APT1 cysts was smaller than the size of cysts from the Pru cell line (Fig. 3*G*). Moreover, to assess the viability of these bradyzoites, we performed plaque assays with parasites recovered from these ex vivo cysts and observed many fewer and much smaller plaques with pSAG1-APT1 parasites than with the Pru parental cell line (Fig. 3 *H–J*). Finally, parasites recovered from in vivo cysts were left for 3 h to invade HFFs (human foreskin fibroblast) and checked by IFA for the expression of APT1-GFP or the Cpn60 apicoplast marker (*SI Appendix*, Fig. S6) and while very few were still expressing the APT1 signal (in line with the spectral cytometry quantifications), all were found to retain the Cpn60 signal. Importantly, this suggests that while almost all bradyzoites lost the expression of APT1, only the few not displaying a severe perturbation of the apicoplast were viable enough to invade, highlighting the importance of the organelle for bradyzoite fitness.

In conclusion, while in an in vivo setting, pSAG1-APT1 tachyzoites induce a more severe acute phase and lead to a higher parasitemia in the brain, the phenotype of brain-derived pSAG1-APT1 parasites regarding cyst size, loss of the apicoplast or overall viability was very similar to the results we obtained with in vitro-generated parasites. Our data thus indicate that the specific loss of APT1 function in in vitro- or in vivo-derived bradyzoites affects apicoplast homeostasis, bradyzoite development, and viability. This suggests that the apicoplast plays an important role for the fitness of bradyzoites.

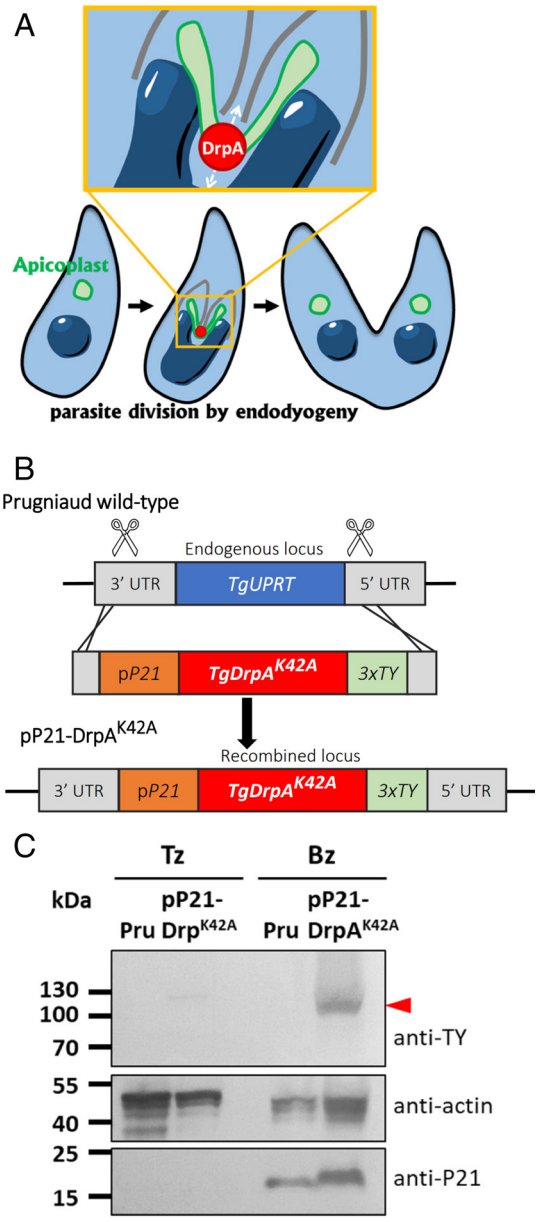

Fig. 4. Generating a parasite line expressing a bradyzoite-specific dominant-negative DrpA mutant. (*A*) Schematic representation of DrpA function in apicoplast fission and inheritance during parasite division. (*B*) Scheme describing the strategy to edit the *UPRT* locus by homologous recombination, in order to introduce a mutated version of the *DrpA* gene and express the DrpA^{K42A} protein under the dependence of a bradyzoite-specific *P21* promoter. (*C*) Immunoblot analysis of protein extracts from tachyzoites (Tz) and 6 d in vitro-differentiated (apicidin treatment) bradyzoites (Bz) with an anti-TY antibody to detect the expression of the DrpA^{K42A} protein (arrowhead) under the dependence of the *P21* promoter. Actin was used as a loading control and P21 as a differentiation control.

Generating a Structural Mutant of the Apicoplast through a Dominant-Negative Approach. Although the exact nature of the metabolic requirement of bradyzoites is unknown, APT1 likely has an important role in the metabolic contribution of the apicoplast for the development of these parasite stages. Yet, as the inhibition of metabolic vs. housekeeping functions of the organelle can lead to different phenotypic outcomes (47), we sought to generate a second apicoplast mutant that would be affected in its ability to divide rather than to perform a particular metabolic function. To this end we targeted DrpA, a dynamin-related protein that was previously shown to be essential for apicoplast fission and inheritance in dividing tachyzoites (Fig. 4*A*) (48). A single mutation in the sequence of the DrpA protein (changing a lysine to an alanine in the GTP-binding site) has been shown to disrupt dynamin function in a dominant-negative fashion in *T. gondii* tachyzoites (48).

We thus designed a strategy to specifically express the mutant version of DrpA (DrpA^{K42A}) in bradyzoites, by using CRISPR/Cas9 to insert the sequence coding for DrpA^{K42A} at the *uracil phosphoribosyl transferase* (*UPRT*) locus (Fig. 4*B*), which is dispensable for bradyzoites in normal culture conditions and for cystogenesis in vivo (49), but allows selection of transgenic parasites with fluorodeoxyribose (50). To drive the expression of the dominant-negative version of DrpA selectively in bradyzoites, we

chose the bradyzoite-specific promoter of P21, a late marker of bradyzoite differentiation (51) that is suitable for this type of strategy (52). Our construct also allowed the addition of a TY epitope tag (53) for subsequent immunodetection of the mutated DrpA version. Transgenic parasites, named pP21-DrpA^{K42A}, were verified by PCR (*SI Appendix*, Fig. S7) and used for in vitro bradyzoite conversion. The UPRT protein is involved in pyrimidine salvage, which can be compensated by de novo synthesis in the parasite (54), but this synthesis relies on CO_2, which has to be omitted from the classical alkaline pH stress differentiation protocol. We thus used treatment with the histone deacetylase inhibitor apicidin (55, 56) as an alternative for initiating in vitro bradyzoite conversion of the pP21-DrpA^{K42A} cell line. Immunoblot analysis showed that the TY-tagged DrpA^{K42A} protein is specifically expressed in in vitro-differentiated bradyzoites (Fig. 4*C*). This was confirmed by immunofluorescence microscopy (Fig. 5*A*), showing that the DrpA^{K42A} protein was essentially absent from tachyzoites, while it was expressed in differentiated parasites. There, it localized to the cytoplasm and to discrete subcompartments of the apicoplast as expected (Fig. 5*A*) (48).

Dominant-Negative pP21-DrpA^{K42A} Parasites Have Their Apicoplast Specifically Affected at the Bradyzoite Stage but Show No Fitness Defect as Tachyzoites. Upon differentiation using apicidin, detailed analysis of DrpA^{K42A}-expressing bradyzoites revealed that they display a reticulated apicoplast labeling or absence of the organelle (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S8), which is similar to what was previously described upon DrpA^{K42A} expression in tachyzoites (48). Apicidin triggered stage conversion very efficiently, but long-term incubation may have unspecific effects, so we also used alternative methods of differentiation to drive DrpA^{K42A} expression and confirmed apicoplast segregation and reticulation problems within cysts upon short-term alkaline stress (as mentioned above long-term treatment is detrimental in absence of the *UPRT* locus) and heat stress–induced differentiation (57) (*SI Appendix*, Fig. S8*A*). Strikingly, when using an anti-P21 antibody on differentiating pP21-DrpA^{K42A} parasites, we could see that cysts/vacuoles in which the morphological impact on the apicoplast was the most visible were those with a higher expression of P21, confirming that the dominant-negative effect is efficiently regulated by the bradyzoite-specific promoter (*SI Appendix*, Fig. S8*B*). However, when cyst size was measured after apicidin-induced in vitro differentiation, in contrast to the results obtained with pSAG1-APT1 parasites, no significant difference was found in comparison with the control (Fig. 5*C*). This highlights potential differences on the timing of phenotype initiation or on the consequences at the cellular level when affecting the metabolism vs. the ultrastructure of the apicoplast. This also called for long-term assessment of viability the pP21-DrpA^{K42A} parasites in the mouse model. Prior to implementing these in vivo experiments, we assessed the in vitro fitness of the tachyzoite stage of the pP21-DrpA^{K42A} cell line and found no particular problem in their ability to form plaques on fibroblast monolayers (Fig. 5 *D* and *E*). Like described earlier, we also checked by flow cytometry analysis that the pP21-DrpA^{K42A} tachyzoites were capable of infecting dendritic cells (46). Our results showed that pP21-DrpA^{K42A} tachyzoites infect dendritic cells as efficiently as the Pru cell line (Fig. 5*F*).

pP21-DrpA^{K42A} tachyzoites, when injected into mice, induced mass loss in the animals in the timeframe corresponding to the acute phase of infection (i.e., up to 17 d, Fig. 5*G*), hinting that this part of the infection process was occurring normally. However, in contrast to the pSAG1-APT1 cell line, infection with pP21-DrpA^{K42A} tachyzoites did not induce any specific mortality in mice during the acute phase (*SI Appendix*, Fig. S9*A*). Parasite quantification by qPCR showed the presence of parasites in the brain as early as 12 d postinfection, confirming that pP21-DrpA^{K42A} mutant parasites are able to disseminate and invade the brain; yet, very little pP21-DrpA^{K42A} parasites were detected 40 d postinfection (Fig. 5*H*). Importantly, there was no difference in the outcome at 40 d when we increased the inoculum from 100 to 2,000 tachyzoites (Fig. 5*H*). In fact, 40 d postinfection, brain samples from mice infected with the pP21-DrpA^{K42A} cell line contained either very few or no detectable cysts, as quantified by qPCR or estimated by microscopic counts (Fig. 5*H* and *SI Appendix*, Fig. S9*B*). The fitness or invasive capacity of pP21-DrpA^{K42A} tachyzoites is not apparently affected in vitro (Fig. 5 *D*–*F*) and our in vivo data show that they likely generate a normal acute phase and are able to reach the brain (Fig. 5 *G* and *H*); however, long-term persistence is affected as illustrated by the almost complete absence of detectable parasites 40 d postinfection (Fig. 5*H* and *SI Appendix*, Fig. S9*B*). This suggests that long-term perturbation of apicoplast division impacts bradyzoite viability and persistence in vivo.

Discussion

The long-term persistence of encysted *T. gondii* parasites in a large proportion of the human population, particularly in ocular tissue and in the central nervous system, has potentially important consequences on the development of a future pathology. As neither the immune system nor current drug-based approaches seem able to eliminate this developmental stage (31, 58), it is essential to look for therapeutic approaches specifically able to target bradyzoites. Because of its evolutionary history and its metabolic importance, the apicoplast has long been considered a promising drug target in the tachyzoite stage, in which both pharmacological and genetic studies have shown that loss of the organelle, loss of its genome, or loss of its metabolic function result in the death of the parasites. Here, we used two independent and complementary genetic approaches to investigate the importance of the apicoplast in the encysted bradyzoite stage.

While there has been recent progress in the in vitro methods used to initiate stage conversion into bradyzoites (18–20), the complexity and diversity of bradyzoites may not always be fully recapitulated in vitro, so the most canonical way to experimentally generate authentic mature *T. gondii* tissue cysts is currently through murine infections. Regulating gene expression in a cyst-enclosed stage that is mostly confined to neuronal tissues (whose access is restricted by the blood-brain barrier), is a technical challenge as it may not be reached efficiently by compounds commonly used for driving conditional silencing. For this reason, we chose a stage-specific promoter-driven strategy to conditionally control gene expression. Although this does not allow complete control of the timing of protein expression, we and others have successfully used the *SAG1* promoter swap strategy to achieve selective loss of expression of a gene of interest in bradyzoites in the past (36, 59, 60). We also took advantage of stage-specific expression in a complementary approach, this time with the promoter of the late bradyzoite marker P21 (52), to specifically switch on the expression of the dominant-negative mutant version of the DrpA protein. Our data show that the regulation of expression was efficient, both for in vitro and in vivo bradyzoite conversion models. However, quantifications of the effect on the apicoplast morphology or the loss of the organelle, while highlighting a strong impact on the organelle, also showed occasionally some phenotypic differences. For instance, apicoplasts were still present in a few cyst-enclosed pSAG1-APT1 bradyzoites recovered 40 d postinfection (Fig. 3*E* and *SI Appendix*, Figs. S2*D* and S6), in spite

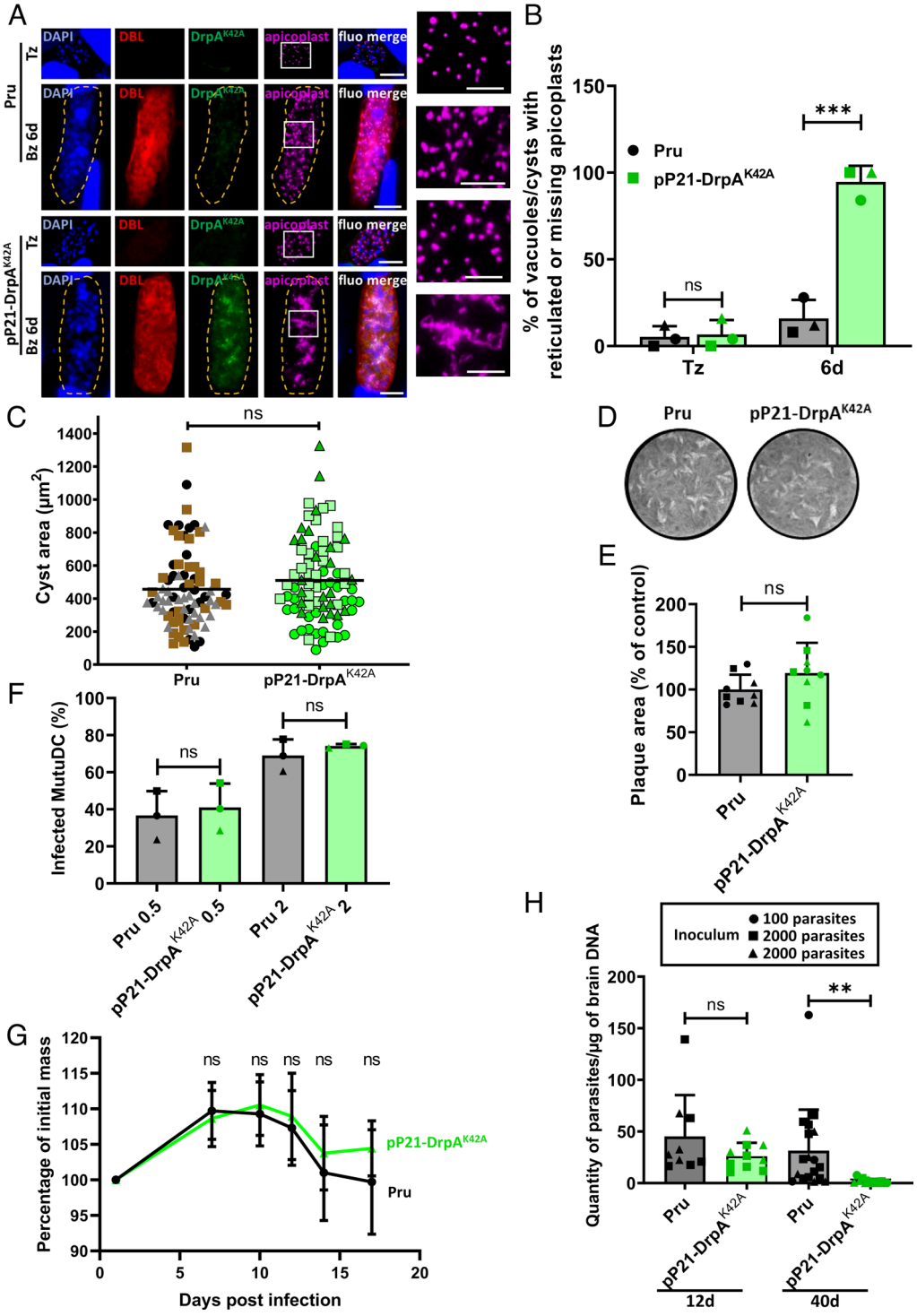

Fig. 5. DrpA^{K42A}-expressing bradyzoites are impacted for apicoplast division and fail to establish chronic phase in mice. (*A*) Upon apicidin-induced in vitro differentiation for 6 d, the DrpA^{K42A} protein is expressed and bradyzoites show extensive reticulation of their apicoplast (Cpn60-labeled, magenta, magnified in *Insets*) in DBL-labeled cysts. The Pru parental cell line and tachyzoites of the two cell lines are shown as a control. Cyst wall is outlined with dashed lines. DNA was stained with DAPI. [Scale bar, 10 µm (5 µm for *Insets*).] (*B*) The proportion of cysts with abnormal apicoplast signal (reticulated or lost) after 6 d of apicidin treatment was quantified. Values are mean from $n = 3$ independent experiments +SD. Symbols are matched between identical experimental groups. ***$P \leq 0.001$, "ns" not significant, unpaired Student's *t* test. (*C*) Quantification of the size of in vitro-differentiated cysts kept for 6 d in the presence of apicidin shows no size difference between cysts of the pP21-DrpA^{K42A} cell line and the Pru control. Data are mean from $n = 3$ independent experiments. Symbols are matched between identical experimental groups. "ns" not significant, nonparametric Mann–Whitney test. (*D*) A representative plaque assay carried out by infecting HFF monolayers with tachyzoites shows that the pP21-DrpA^{K42A} parasites are not affected in their lytic cycle in vitro. (*E*) Measurement of lysis plaque areas confirms that there is no major defect in the lytic cycle of pP21-DrpA^{K42A} tachyzoites. Values are mean from $n = 3$ independent experiments (each one with three technical replicates) +SD. Mean value for the Pru cell line was set to 100% as a reference. Symbols are matched between identical experimental groups. ****$P \leq 0.0001$, unpaired Student's *t* test. (*F*) In vitro dendritic cell infection assay was performed and analyzed by flow cytometry and shows no impairment of the invasive capacity of pP21-DrpA^{K42A} tachyzoites. Data are mean from $n = 3$ independent experiments; "ns" not significant, unpaired Student's *t* test. (*G*) Monitoring of body mass of mice infected with Pru or of pP21-DrpA^{K42A} tachyzoites shows that the acute phase occurs normally with of pP21-DrpA^{K42A} parasites. Data represent mean from 20 individual mice from $n = 2$ independent experiments +SD. "ns" not significant, unpaired Student's *t* test. (*H*) Parasite burden at 12 d or 40 d postinfection measured by qPCR on genomic DNA from infected mice. Values are from $n = 2$ (12 d timepoint) or $n = 3$ (40 d timepoint) independent experiments, performed with an inoculum of 100 parasites or 2,000 parasites, and are expressed as mean +SD. **$P \leq 0.01$, "ns" not significant, unpaired Student's *t* test.

of a very efficient downregulation of APT1 (*SI Appendix*, Figs. S4 *C–E* and S6). Although these APT1-deficient organelles may already not be fully functional, it seems that a stronger effect on the apicoplast occurs later and with some degree of heterogeneity. This is not surprising because stage conversion is a continuum that may happen asynchronously. As a result, there can be some heterogeneity in gene expression, as single-cell RNAseq analyses of in vitro-differentiated parasites suggest (61). Analyses on brain-derived cysts also highlighted a fairly large diversity between cysts and among bradyzoites within the same cyst (5, 62). Our work nevertheless confirms that stage-specific promoters can be used efficiently to investigate gene function in bradyzoites through a simple promoter replacement.

We purposely used different approaches and different types of mutants affected in apicoplast homeostasis, with one primarily impacted for metabolism and the other one in organelle fission and inheritance: We wanted to see if there was converging evidence on the essentiality of the organelle in bradyzoites. Interestingly, the two cell lines we generated through these different approaches were found to display phenotypes that are not completely overlapping (*SI Appendix*, Fig. S10). One particularly striking difference is the strong acute phase induced by the pSAG1-APT1 cell line (Fig. 3 *A–D*). One possible explanation is that promoter replacement likely led to an overexpression of APT1 (*SI Appendix*, Fig. S1 *C* and *D*), and this might in turn have conferred a metabolic and thus a fitness advantage to tachyzoites in the host. On the other hand, our plaque assays show that there does not seem to be an increased fitness for tachyzoites in vitro (Fig. 2*D* and *SI Appendix*, Fig. S5). Discrepancies between in vitro and in vivo settings have been highlighted in several studies describing the metabolic flexibility of *T. gondii* tachyzoites, which are able to scavenge a number of nutrients in rich culture medium but may encounter more restrictive conditions in the animal host (63). A number of host and parasite factors account for the heterogeneity in the ranges of mice brain cyst burden described in the literature, and while it is difficult to establish an exact correlation between the cyst burden and the initial inoculum, it is quite likely that a more successful acute phase can lead to increased parasite load in the brain (64, 65). It is thus possible that a fitness advantage during the acute phase would be the reason of the high cyst numbers generated by the pSAG1-APT1 cell line. These large cyst numbers may also potentially reflect multiple reactivation and reinvasion events, which might also explain the fact that these cysts were generally found to be smaller than those of the parental cell line. However, the majority of pSAG1-APT1 cysts that were recovered showed morphological alterations, especially regarding the apicoplast (Fig. 3 *E* and *D* and *SI Appendix*, Fig. S2). Moreover, bradyzoites that were released from these cysts were found to be largely nonviable (Fig. 3 *H–J*). So, altogether our data rather suggest that the small cysts reflect a problem in parasite development instead of recent parasite establishment. Importantly, no in vivo-generated bradyzoite with a strong apicoplast defect (i.e., lacking apicoplast marker labeling) was fit enough to invade host cells upon release from cysts (*SI Appendix*, Fig. S6), further supporting the importance of the organelle for bradyzoite fitness.

In contrast, the pP21-DrpA^{K42A} cell line largely failed to generate cysts (Fig. 5*H*). We could establish that it was not due to an alteration in the acute phase, as the parasites were found to reach the brain at the end of this phase of infection (Fig. 5 *G* and *H*). Instead, this probably rather highlights an increased elimination by the immune system, or/and a strong defect in cystogenesis. While both our strategies were aimed at interfering with the function of the apicoplast, the different outcomes in the animal model concerning cyst generation may be due to the different promoters that were used, which, respectively, turn genes on or off at different times during the stage conversion process, thus affecting the timeframe of the manifestation of the detrimental phenotypes.

Another main difference in the mutants we generated is that while for both there was a visible morphological impact in vitro, the pSAG1-APT1 mutant cell line was already strongly affected in cyst growth during in vitro differentiation (Fig. 2*C*), while for pP21-DrpA^{K42A} bradyzoites the detrimental effect on cystogenesis only manifested during long-term infection in the animal (Fig. 5 *C* and *H*). This could be related to the fact that the former targets metabolic pathways hosted by the organelle, while the latter targets its division and inheritance in parasite progeny. It has been known for some time that there are differences in the kinetics of parasite demise whether a metabolic pathway of the apicoplast or a housekeeping function of the organelle are targeted (47). Targeting apicoplast-hosted metabolism through genetic or pharmaceutical-based approaches usually lead to a rapid death of the parasites, while affecting apicoplast division usually leads to a so-called delayed death, where parasites are only affected after another round of reinvasion. While in vitro-differentiated pP21-DrpA^{K42A} bradyzoites showed marked signs of morphological alteration of their apicoplasts (Fig. 5*A* and *SI Appendix*, Fig. S8), the remaining organelles potentially retained some metabolic capacity. A strong impact on the apicoplast, which is linked to organelle inheritance defects in this mutant, likely depends on the parasites undergoing several successive rounds of division that would only happen over a longer period of time. It was shown that *T. gondii* tachyzoites can potentially share resources (metabolites and proteins) through a connection at their basal pole, ensuring proper cellular division in spite of an absence of apicoplast of several parasites within the PV, and thus explaining the delayed death effect (66). However, bradyzoite division is largely asynchronous and there is no connection between the parasites in mature cysts (9, 66, 67), which suggests that organelle loss may be more detrimental for this developmental stage. Moreover, this might be particularly important in an in vivo setting, where nutrient sources are potentially scarce, which could explain why cystogenesis of pP21-DrpA^{K42A} parasites ended up being strongly affected in these conditions.

Importantly, in the mouse model, both pSAG1-APT1 parasites (that generate numerous but small cysts, containing less fit bradyzoites) and pP21-DrpA^{K42A} parasites (that display a strong defect in cystogenesis) showed converging evidence that the apicoplast is important for *T. gondii* survival and persistence as bradyzoites. This now raises the question of which essential function(s) the organelle is performing for this developmental stage. An earlier in vivo morphological study showed that duplication or elongation of the apicoplast could still be observed in bradyzoites many weeks or even months postinfection, suggesting an active role for the organelle (68). Due to the lack of functional studies, the metabolic activities and requirements of bradyzoites are, however, still largely uncharacterized. RNA expression data from in vivo-generated cysts suggest that the four main metabolic pathways hosted by the apicoplast could be active in bradyzoites, with enzymes of the iron–sulfur cluster and isoprenoid synthesis pathways even potentially up-regulated (16). It would now be particularly interesting to apply our conditional strategies to specifically interfere with individual enzymes of these pathways in bradyzoites.

While finely tuned control of protein expression is technically difficult to achieve directly in brain-encysted mature bradyzoites, through this work we have provided corroborating lines of evidence showing that the organelle is important for bradyzoite fitness and persistence. Importantly, this validates the organelle as a potential drug target in the context of the establishment of chronic

toxoplasmosis. Of course, it should be kept in mind that designing drugs able to interfere with the apicoplast in bradyzoites will constitute a major challenge. For instance, long-term treatment in mice suggests that apicoplast-targeting drugs like clindamycin and spiramycin can both reduce cyst burden, yet they may not completely eradicate the parasites (69, 70), and compounds targeting a specific pathway hosted by the organelle would have to be able to go through a series of physical hurdles that include the blood–brain barrier, the host cell plasma membrane, the cyst wall, the parasite plasma membrane and, finally, the four membranes of the organelle. Also, as bradyzoites are slow growing, they are likely to be less sensitive to metabolic targeting, by analogy with bacterial or tumoral "persister" cells that are notoriously prone to resist to drugs (71). However, although cyst-enclosed bradyzoites display some heterogeneity in their cellular and metabolic activities, they remain dynamic growing entities (62), and our in vivo results suggest that they are likely to be vulnerable to long-term interference with apicoplast-related functions. So, the possibility to exploit this as a therapeutic strategy should not be overlooked.

Materials and Methods

Animal Care and Ethics Statement. Animal care and use protocols were carried out under the control of the National Veterinary Services and in accordance with the European Union guidelines for the handling of laboratory animals EEC Council Directive, 2010/63/EU, September 2010). The protocol inducing pain (APAFIS#25130-2020040721346790 v3) was approved by the local Ethical Committee for Animal Experimentation registered by the "Comité National de Réflexion Ethique sur l'Expérimentation Animale" under no. CEEA122. CBA/J mice were purchased from Janvier Labs (France).

Parasites and Cells Culture. Tachyzoites of the Pru *T. gondii* strain (72), as well as derived transgenic parasites, were maintained by serial passage in monolayers of HFF (American Type Culture Collection, CRL 1634) grown in Dulbecco's modified Eagle medium (Gibco), supplemented with 5% of decomplemented fetal bovine serum, 2 mM glutamine, and a cocktail of penicillin–streptomycin at 100 µg/mL.

In Vitro Differentiation. In vitro conversion into bradyzoites was achieved either through alkaline pH stress (57), or by treating with the histone deacetylase inhibitor apicidin (55), or by heat-stress (57) (*SI Appendix, Materials and Methods*).

Mouse Infection, Cyst Purification, and Bradyzoite Isolation. Seven-week-old male CBA/J mice (Janvier Labs) were infected intraperitoneally with 100 or 2,000 tachyzoites of the appropriate strains. At different timepoints postinfection, the brains were collected and cysts were isolated by isopycnic centrifugation (73) and bradyzoite were recovered after pepsin treatment. Parasite quantification was performed as described previously (60) (*SI Appendix, Materials and Methods*).

Additional Methods and detailed protocols are provided in *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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