Role of amylopectin synthesis in *Toxoplasma gondii* and its implication in vaccine development against toxoplasmosis

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*Toxoplasma gondii* is a ubiquitous pathogen infecting one-third of the global population. A significant fraction of toxoplasmosis cases is caused by reactivation of existing chronic infections. The encysted bradyzoites during chronic infection accumulate high levels of amylopectin that is barely present in fast-replicating tachyzoites. However, the physiological significance of amylopectin is not fully understood. Here, we identified a starch synthase (SS) that is required for amylopectin synthesis in *T. gondii*. Genetic ablation of SS abolished amylopectin production, reduced tachyzoite proliferation, and impaired the recrudescence of bradyzoites to tachyzoites. Disruption of the parasite Ca$^{2+}$-dependent protein kinase 2 (CDPK2) was previously shown to cause massive amylopectin accumulation and bradyzoite death. Therefore, the Δcdpk2 mutant is thought to be a vaccine candidate. Notably, deleting SS in a Δcdpk2 mutant completely abolished starch accrual and restored cyst formation as well as virulence in mice. Together these results suggest that regulated amylopectin production is critical for the optimal growth, development and virulence of *Toxoplasma*. Not least, our data underscore a potential drawback of the Δcdpk2 mutant as a vaccine candidate as it may regain full virulence by mutating amylopectin synthesis genes like SS.

1. Background

*Toxoplasma gondii* is an obligate intracellular parasite of the protozoan phylum Apicomplexa that comprise many parasitic pathogens of medical and veterinary importance, such as *Plasmodium* and *Eimeria* species. Infections by *Toxoplasma* are highly prevalent in humans and animals [1,2]. One factor contributing to the wide spread of *T. gondii* is the multiple routes of transmission [3,4]. Oocysts shed by cats in the environment serve as a major source of infection to new hosts [5]. *Toxoplasma* can also be transmitted between intermediate hosts that include many warm-blooded animals [6–8]. For example, humans may get infected by consuming undercooked meat infected with *Toxoplasma* cysts [9]. *Toxoplasma gondii* exists in two asexual forms in its intermediate hosts. A fast-replicating tachyzoite form that underlies acute infection and associated clinical symptoms, and a slow-growing bradyzoite form that causes persistent, usually life-long, chronic infection [4,10]. Depending on the environmental cues, tachyzoites and bradyzoites can interconvert, which is crucial for the pathogenesis and transmission of *T. gondii* [11]. Nonetheless, the molecular mechanisms that govern such conversions remain largely elusive.
The encysted bradyzoites and oocysts contain abundant amylopectin granules, which are barely apparent in tachyzoites [12–16]. Such granules were first described over 50 years ago [17]; however, their metabolic pathways and biological significance are not yet well-examined experimentally. It is widely assumed that amylopectin is a rich source of energy for bradyzoites and oocysts, because they are enclosed by a thick wall that is poorly permeable to external nutrients [12]. It is also proposed that amylopectin may provide energy to bradyzoites and oocysts during their conversion to tachyzoites [18]. Recently, a CPDK2 in the parasite was shown to regulate the amylopectin production, likely through phosphorylation of vital enzymes involved in amylopectin synthesis and/or degradation [19,20]. The absence of CPDK2 causes tachyzoites and bradyzoites to accumulate massive amounts of amylopectin. As a probable consequence, the Δcdpk2 mutants fail to produce tissue cysts [20]. Amylopectin accumulation in the Δcdpk2 strain can be reversed by a point mutation (changing Ser 25 to Glu, which results in a hyperactive enzyme) in glycogen phosphorylase—an enzyme involved in amylopectin degradation [20]. These studies demonstrated the importance of amylopectin metabolism in parasites, though the exact physiological roles of amylopectin are still unclear. Our work focused on a starch synthase (SS) that we show is involved in amylopectin biosynthesis in T. gondii.

2. Methods

2.1. Parasite strains and growth in vitro

The ME49 strain of T. gondii and its derivative strains were propagated in human foreskin fibroblasts (ATCC no. SCRC-1041, USA), which were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Life Technologies, USA), 100 μg ml⁻¹ streptomycin and 10 mM L-glutamine [21].

2.2. Construction of SS and CDPK2 mutants and phenotyping

Mutants lacking SS and/or CDPK2 were constructed by CRISPR/Cas9-assisted homologous gene replacement. Gene-specific CRISPR plasmids were generated by site-directed mutagenesis (using primers listed in electronic supplementary material, table S1), as previously described [22,23]. The donor DNA templates were constructed by cloning the 5’- and 3’-homology arms, flanking a drug selection marker (DHFR or CAT), into the pUC19 vector using the ClonExpress II One-Step cloning kit (Vazyme Biotech, China) [21]. The gene-specific CRISPR plasmids and homologous donor templates were co-transfected into extracellular tachyzoites of the ME49 or derivative strains, followed by selection with 1 μM pyrimethamine (Sigma-Aldrich, USA) or 30 μM chloramphenicol (Sigma-Aldrich, USA), and parasite cloning by limiting dilution. The positive mutant clones were identified by diagnostic PCRs (PCR1–3, using primers in electronic supplementary material, table S1). Plaque, replication and bradyzoite differentiation assays were performed using protocols reported earlier [24,25].

2.3. Western blotting

To generate antibodies against SS, a 6xHis-tagged polypeptide corresponding to the SS fragment from E136 to L587 was expressed and purified from E. coli. The recombinant protein was used to immunize rabbits for the production of polyclonal antibodies, which were tested by Western blotting as previously described [26]. Briefly, about 4 × 10⁷ parasites were lysed in 40 μl of SDS-sample buffer, of which 20 μl was loaded and resolved by 4–12% gradient SDS-PAGE gels, followed by protein transfer to nitrocellulose membranes and immunoblotting with rabbit anti-SS sera. The rabbit anti-TgALD antibody (provided by Dr David Sibley, Washington University in St Louis) was included as a loading control. Primary antibodies were detected by HRP conjugated goat anti-rabbit IgG (Boster Biological Technology, China). The blots were then developed by the ECL kit (Thermo Fisher Scientific, USA) and subsequently scanned by the Amersham Typhoon NIR imager (GE Healthcare, USA).

2.4. Periodic acid–Schiff staining

To stain tachyzoites, extracellular parasites were used to infect fresh HFF monolayers seeded on glass coverslips and cultured for 24 h. Then the samples were stained with periodic acid–Schiff (PAS) [26]. To stain bradyzoites, parasite cultures were subjected to alkaline stress (culture medium with pH = 8.2, ambient CO₂) for 5 days in T25 flasks. Subsequently, syringe-released parasites were used to infect fresh HFF cells on coverslips and cultured under the same alkaline conditions for 4 days prior to staining [19]. All samples were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (Sigma-Aldrich, USA) and stained with Hoechst 33342 (Beyotime, China) and/or FITC-conjugated Dolichos biflorus agglutinin (DBA-FITC) (Vector Laboratories, USA). Successive PAS staining was performed by a standard procedure reported previously [19]. Briefly, samples were incubated in 1% periodic acid (Sigma-Aldrich, USA) for 5 min, washed with tap water for 1 min, and rinsed once with distilled water. Cells were then incubated with Schiff’s reagent (Sigma-Aldrich, USA) for 15 min, washed with tap water for 10 min, and rinsed three times with PBS. Cultures were imaged by the Olympus BX53 microscope (Olympus, Japan) equipped with an Axiocam 503 mono camera (Carl Zeiss, Germany).

2.5. Metabolic labelling

Fresh extracellular tachyzoites (3 × 10⁷) were incubated in DMEM medium containing 8 mM ¹³C₆-glucose (37°C, 4 h). Subsequently, the parasites were washed three times with glucose-free DMEM and resuspended in 50% aqueous methanol. Metabolites were extracted using established protocols and analysed by UHPLC-HRMS (ultra-high-performance liquid chromatography high-resolution mass spectrometry) [26]. Chromatographic separation was performed on a UHPLC system (Thermo-Fisher UltiMate 3000, Thermo Fisher Scientific, USA) with a Waters BEH Amide column (2.1 × 100 mm, 1.7 μm). The injection volume was 5 μl and the flow rate was adjusted to 0.35 ml min⁻¹ with a linear gradient elution. The mobile phases consisted of water (phase A) and acetonitrile/water (90:10, v/v) (phase B). Both phases contained 10 mM ammonium formate (pH = 9.0). The eluents were analysed on a mass spectrometer (Thermo-Fisher Q Exactive Hybrid Quadrupole-Orbitrap).
using the HESI (heated electrospray ionization) negative mode. A high-resolution scan was obtained (70–400 m/z) with AGC (automatic gain control) target set as 3 × 10⁶. The m/z spectra and relative abundance of metabolites were analysed by Xcalibur (v.4.0.27.19) (Thermo Fisher Scientific, USA).

2.6. Virulence tests and counting of parasite cysts in mice brains

Female ICR mice (7 weeks old) were subjected to intraperitoneal injection with purified tachyzoites of indicated strains (100 parasites/mouse), and animals were subsequently monitored daily for 30 days. An indirect ELISA test that detects TgMIC3 specific antibodies was used to determine the infection status of the surviving mice. Seropositive mice were anaesthetized and sacrificed to isolate the brain tissues, which were then homogenized and stained with DBA-FITC to determine the number and size of Toxoplasma cysts, as described [27]. All animal experiments were approved by the Scientific Ethics Committee of Huazhong Agricultural University (permit no. HZAUMO-2018-034).

2.7. Phylogenetic analysis

Protein sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov). Sequences were aligned by Clustal W and curated to remove low-homology regions. The phylogenetic tree was constructed using the maximum-likelihood method based on the JTT matrix-based model in MEGA 7.0. Bootstrap analysis was performed with 1000 replicates. Finally, a tree based on the JTT matrix-based model in MEGA 7.0. Bootstrap tree was constructed using the maximum-likelihood method to determine the number and size of Toxoplasma cysts, as described [27]. All animal experiments were approved by the Scientific Ethics Committee of Huazhong Agricultural University (permit no. HZAUMO-2018-034).

3. Results

3.1. Construction of a starch synthase deficient mutant

We first searched for the enzymatic pathways that may catalyse amylopectin synthesis and degradation in T. gondii. The enzymes involved in starch metabolism in Arabidopsis and glycerogen metabolism in humans were used as baits, to identify homologous proteins in the Toxoplasma genome. Hits from Blast searches and sequence analyses were used to construct a putative network involved in amylopectin metabolism in T. gondii (figure 1a,b). Our predicted pathways were similar to what has been proposed before [19], although small differences do exist. For example, we predicted that the gene TgME49_271210 encoded a disproportionating enzyme, which may contribute to both the synthesis and degradation of amylopectin. Whereas others suggested that it is a debranching enzyme involved in amylopectin degradation [19].

2.8. Statistical analysis

Statistical analyses were performed in Prism 7 (GraphPad Software, USA), using Student’s t-test, one-way ANOVA, two-way ANOVA or Gehan–Breslow–Wilcoxon test, as indicated in pertinent figure legends.

3.2. Starch synthase is critical for amylopectin synthesis and its inactivation leads to reduced utilization of exogeneous glucose

To examine the role of SS during amylopectin synthesis, the Δss mutant was subjected to PAS staining of polysaccharides. Under standard growth condition, the ME49 tachyzoites were weakly stained, suggesting a mild accumulation of amylopectin. By contrast, no visible PAS signal was detected in the Δss mutant (figure 2c). To confirm the disruption of SS, we generated rabbit antisera and performed immunoblotting. In the parental ME49 strain, a protein band above 245-kDa was observed, which is consistent with the theoretical molecular weight of SS (330-kDa). This band was not detectable in the Δss mutant (figure 2c), suggesting successful deletion of SS.
Signals in the ME49 parental strain were notably brighter than those in tachyzoites. Likewise, the Δcdpk2 mutant exhibited massive amounts of amylopectin in alkaline-induced cultures. However, the Δss mutant did not show any PAS staining signal even under bradyzoite-inducing conditions (figure 2d; electronic supplementary material, figure S2a), further confirming that SS is needed for amylopectin synthesis in Toxoplasma. Amylopectin levels in ME49 and the Δss mutant were also determined by HPLC after being degraded to glucose by α-amylase and α-glucosidase. Although there was variation among experiments, our results repeatedly showed that the amylopectin level in the Δss mutant was lower than that in the parental strain ME49, at both the tachyzoite and the bradyzoite stages (electronic supplementary material, figure S2b).

We next investigated whether the SS deletion affected the catabolism of glucose, as it is the major substrate for amylopectin synthesis, as well as an important energy source for the parasites [30]. In this regard, we labelled the fresh extracellular parasites with 13C6-glucose for 4 h and measured the inclusion of isotopic carbon into glycolysis and TCA-cycle intermediates by mass spectrometry. Surprisingly, we observed that flux of 13C into glycolysis (glucose-6-phosphate, fructose-6-phosphate, pyruvate and lactate), TCA cycle (succinate and malate), pentose phosphate pathway (sedoheptulose-7-phosphate) and certain amino acids (glutamate and aspartate) was significantly decreased in the SS-knockout mutant when compared to the parental strain (figure 3). Moreover, much less cellular glucose was labelled with 13C in the mutant, suggesting reduced import of glucose into parasites from the medium. The reduced efficiency of glucose catabolism in the Δss mutant is consistent with its slower growth (see below). Although it is not clear whether reduced glucose catabolism is directly causing the growth defect of Δss mutant, these data indicate that robust uptake and utilization of exogenous glucose requires a functional SS. The fact that the Δss mutant was less efficient in using exogenous glucose prompted us to check whether it relied more on glutamine, another important carbon source for Toxoplasma parasites. In medium containing glucose but no

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**Figure 1.** Enzymes involved in amylopectin metabolism in *T. gondii* based on *in silico* analysis. (a) Pathways of amylopectin synthesis and degradation. (b) Proteins mediating each reaction in (a) and their corresponding gene IDs. (c) Phylogenetic relationships of SS homologs from selected organisms. Protein sequences were retrieved from NCBI and the phylogenetic tree was constructed using the maximum-likelihood method (MEGA7).
glutamine, the Δss mutant had similar degree of growth reduction as that seen in medium containing both glucose and glutamine (electronic supplementary material, figure S3). Surprisingly, when both glucose and glutamine were taken away from the medium, the Δss mutant proliferated significantly faster than the parental strain ME49 (electronic supplementary material, figure S3). The underlying basis for the increased replication rate of the Δss mutant in the glucose

Figure 2. Amylopectin accumulation in the tachyzoites and bradyzoites of wild-type and mutant strains. (a) Genetic deletion of SS by double homologous recombination, mediated by a dual-gRNA CRISPR gene editing system. PCR1–3 are products of diagnostic PCRs used to identify the clonal mutants. (b) Diagnostic PCRs on a representative Δss clone. (c) Immunoblot confirming the loss of SS expression in the Δss mutant, ALD served as a protein loading control. (d) PAS staining of amylopectin in the parasites. Indicated strains were cultured under tachyzoite growth condition for 24 h, or under bradyzoite-inducing conditions (pH = 8.2, ambient CO2) for 9 days. Samples were subjected to DBA-FITC, PAS and Hoechst staining to visualize the cyst wall, amylopectin granules and nuclei, respectively.

Figure 3. Utilization of exogenous glucose determined by metabolic tracing. Tachyzoites of the ME49 (WT) and Δss (KO) strains were incubated in DMEM medium containing 8 mM 13C6-glucose for 4 h, and incorporation of 13C into selected metabolites was determined by UHPLC-HRMS. Means ± s.e.m. of three independent experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, two-way ANOVA. F6P, fructose-6-phosphate; S7P, sedoheptulose-7-phosphate; G6P, glucose-6-phosphate; Pyr, pyruvate; Lac, lactate; Suc, succinate; Mal, malate; Glu, glutamate; Asp, aspartate; G3P, glycerol-3-phosphate; Thr, threonine. M0–M7 denotes the number (0–7) of carbons labelled with 13C in the corresponding molecules.
and glutamine deficient medium is currently unknown, but it further suggests an altered metabolic capacity or need of this mutant.

3.3. Inactivation of starch synthase impairs the tachyzoite growth

In further experiments, we performed plaque assays in prolonged (two weeks) unperturbed cultures to examine the parasite fitness. The plaques formed by the Δss mutant were significantly smaller than those of the parental ME49 strain (figure 4a,b), while the number of plaques was indistinguishable, suggesting a slower growth of the mutant. Next, we tested the proliferation rate of the Δss mutant. In this regard, parasites were allowed to invade host cells for 1 h and then replicate for an additional 24 h. Subsequently, the number of parasite progeny in each parasitophorous vacuole (PV) was determined (figure 4c). Consistent with our plaque assays, the Δss mutant replicated significantly slower than the parental strain, as judged by fewer big vacuoles (with greater than eight tachyzoites) and more small vacuoles (less than four parasites). Our attempts to complement the Δss mutant were futile, likely due to the large size of this gene (genomic sequence greater than 34 kb). Nonetheless, we examined the phenotype of two independent Δss clones (electronic supplementary material, figure S4a,b), both of which exhibited defect in amylopectin production and growth as described above, affirming that the indicated phenotypes were caused by inactivation of SS.

3.4. Inability to synthesize amylopectin impairs the reactivation of bradyzoites to tachyzoites

The fact that bradyzoites accumulate amylopectin granules and we observed a reduction of starch synthesis (see above) in the Δss mutant prompted us to test the role of SS during bradyzoite differentiation. We cultured the ME49 and Δss strains in alkaline medium (pH = 8.2) and ambient CO2 to induce bradyzoite formation, followed by DBA-FITC that stains the cyst wall. After 4 days induction, a modest but significant increase in the differentiation rate of all three independent Δss mutant clones (figure 4d; electronic supplementary material, figure S4c) was observed. For comparison, both strains had similar and very low levels of bradyzoite differentiation under normal growth conditions (pH = 7.2, 5% CO2), suggesting no significant spontaneous conversion of the Δss mutant. Because amylopectin may serve as an energy source during reactivation of bradyzoites into tachyzoites, we tested the hypothesis whether defects in amylopectin synthesis would impair the reactivation process using the Δss mutant. Cultures were incubated with alkaline medium and ambient CO2 for 12 days to enrich bradyzoites, and then the medium and growth conditions were reverted for normal tachyzoite cultivation to monitor the bradyzoite-to-tachyzoite conversion. As illustrated by DBA staining (figure 5), bradyzoites formed by the parental strain gradually switched to tachyzoites upon change to standard tachyzoite growth condition. Within 36 h, the fraction of DBA-positive vacuoles (indicative of bradyzoites) was reduced from 71.66 to 20.4%, in strong contrast to the Δss mutant, which displayed only a modest decline in cyst staining from 86 to 72.46% within

![Figure 4. Growth and development of the Δss mutant in vitro.](image)

**Figure 4.** Growth and development of the Δss mutant in vitro. (a) Growth of the ME49 or Δss tachyzoites, as determined by plaque assays. Scale bar = 2 mm. (b) Relative size of the plaques formed by ME49 versus Δss strains cultured in HFF monolayers for 14 days. Means ± s.e.m., **p < 0.01, Student’s t-test. (c) Intracellular replication rates of the indicated strains, as determined by the fraction of vacuoles containing 1, 2, 4, 8 and 16 or more parasites. Means ± s.e.m. of three independent experiments, ***p ≤ 0.001, two-way ANOVA. (d) Bradyzoite differentiation rates of ME49 versus the Δss strains. Parasite cultures were induced in alkaline media for 4 days before DBA-FITC staining to determine the efficiency of bradyzoite transition. Means ± s.e.m. of three independent experiments, *p < 0.05, Student’s t-test.

![Figure 5. SS is important for the reactivation of bradyzoites in vitro.](image)

**Figure 5.** SS is important for the reactivation of bradyzoites in vitro. ME49 and Δss parasites were first cultured in the alkaline medium at ambient CO2 for 12 days to form bradyzoites (DBA-positive). The medium was then changed to favour tachyzoite growth at 5% CO2 for indicated periods, and samples were subjected to DBA-FITC staining. The decrease in DBA-positive vacuoles implies bradyzoite reactivation. Means ± s.e.m. of three independent experiments.
although impaired in amylopectin synthesis, the surviving animals (30 days post-infection). Surprisingly, mutant cysts was higher and more heterogeneous (figure 6a).

The starch synthase mutant displays heterogeneous tissue cysts in vivo

To investigate the importance of amylopectin synthesis in vivo, we infected mice with the \( \Delta ss \) mutant and parental strains and monitored the survival of infected animals. Despite a mild growth defect in vitro, the virulence of the \( \Delta ss \) mutant was indistinguishable from that of the ME49 strain (figure 6a). We also examined the cyst burden in surviving animals (30 days post-infection). Surprisingly, although impaired in amylopectin synthesis, the \( \Delta ss \) mutant produced a similar number of tissue cysts to the parental strain (figure 6b). However, the mean size (diameter) of the mutant cysts was higher and more heterogeneous (figure 6c), which suggests that amylopectin synthesis contributes to maintaining the regular size of tissue cysts.

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3.6. SS deletion abolishes amylopectin accumulation in the \( \Delta cdpk2 \) mutant and can restore its virulence and cyst formation

Previous work has shown that deletion of CDPK2 in T. gondii leads to starch accumulation and is lethal to bradyzoites [20]. It is not clear however, whether the starch accrual underlies the observed phenotype. To address this question, we constructed the \( \Delta cdpk2 \) and \( \Delta ss-\Delta cdpk2 \) mutants (figure 7a,b). Similar to the \( \Delta ss \) strain, both \( \Delta cdpk2 \) and \( \Delta ss-\Delta cdpk2 \) mutants displayed a modest but significant reduction in plaque size (figure 7c,d). PAS staining confirmed that CDPK2 inactivation caused massive accumulation of amylopectin in tachyzoites as well as bradyzoites (figure 7e; electronic supplementary material, figure S2a), which was completely abolished upon deletion of SS (\( \Delta ss-\Delta cdpk2 \)), further confirming a role of SS in amylopectin synthesis.

Using our single and double mutants, we determined the importance of amylopectin synthesis during host infection. In the virulence test (figure 6a), the \( \Delta cdpk2 \) mutant displayed a modest attenuation as judged by the higher survival rate (55%) of mice infected with this mutant than the parental ME49 strain (23.8%). Quite notably however, the \( \Delta ss-\Delta cdpk2 \) double mutant exhibited normal virulence (figure 6a). Moreover, consistent with the previous report [20], the mice that survived the infection by the \( \Delta cdpk2 \) mutant did not harbour any cysts in their brain (figure 6b) [20]. However, the cyst formation in the \( \Delta ss-\Delta cdpk2 \) double mutant was similar to the parental strain. Similarly, the size distribution of the brain cysts derived from the \( \Delta ss-\Delta cdpk2 \) mutant was indistinguishable from that of the wild-type strain (figure 6c). In brief, our results show that SS disruption not only reversed the virulence defects of the \( \Delta cdpk2 \) mutant but also restored its cyst formation capacity.

4. Discussion

Amylopectin granules are a hallmark of the bradyzoite and oocyst stages, which distinguish them from the fast-replicating tachyzoite stage of T. gondii [16]. In this study, we predicted the underlying metabolic pathways, and revealed that SS is essential for amylopectin production. Mutants lacking SS are unable to synthesize amylopectin in either tachyzoite or bradyzoite stages. Moreover, deletion of SS in the \( \Delta cdpk2 \) strain completely reversed the amylopectin accumulation phenotype of the latter mutant, further confirming a role of SS in starch synthesis. The \( \Delta ss \) mutant therefore allowed us to assess the importance of amylopectin in parasites. Our results demonstrate that SS is required for optimal growth of tachyzoites, likely by promoting the utilization of exogenous glucose. Importantly, we show that amylopectin synthesis is critical for the reactivation of
bradyzoites to tachyzoites under favourable conditions. Mutants lacking SS were able to differentiate to bradyzoites in vitro as well as in vivo. At least in the in vitro model, bradyzoites of the Δss mutant did not respond to the reactivation signals effectively and were reluctant to convert to tachyzoites. This is the first genetic evidence to the best of our knowledge revealing that amylopectin may contribute to the reactivation of chronic toxoplasmosis.

Amylopectin accumulates in selected life cycle stages of coccidian parasites. Its physiological function has never been fully defined however. Early studies have shown that treating Eimeria or Cryptosporidium oocysts with high temperature (35°C or above) gradually depletes amylopectin and decreases the infectivity and durability of oocysts [31,32]. It was proposed that amylopectin may serve as a reservoir of energy in oocysts or tissue cysts. This hypothesis has never been tested by genetic approaches. Our T. gondii Δss mutant defective in amylopectin synthesis offered an opportunity to test this. While the Δss mutant was able to form bradyzoites upon induction with stress conditions, its bradyzoite to tachyzoite conversion was impaired after changing the cultures back to tachyzoite growth conditions. We still do not understand how exactly SS and amylopectin help recrudescence. The metabolic tracing experiments indicate that even in tachyzoites, which do not accumulate large amounts of amylopectin, SS deletion reduced the uptake and utilization

Figure 7. Construction and characterization of the Δss-Δcdpk2 double mutant. (a) Illustration of replacing the CDPK2 gene by a CAT selection marker in the Δss mutant. (b) Diagnostic PCRs of a representative clonal Δss-Δcdpk2 mutant. (c) Growth of the shown strains, as determined by plaque assays. (d) Relative size of the parasite plaques formed in HFF monolayers. Means ± s.e.m., **p ≤ 0.001, one-way ANOVA comparing the indicated strains to ME49. (e) Starch accumulation in the Δss-Δcdpk2 mutant, as determined by PAS staining.
of exogenous glucose (figure 3). These data suggest that some of the imported glucose is converted to glucans and then degraded to enter glycolysis and TCA cycle in tachyzoites. A dynamic amylopectin synthesis and breakdown may allow the parasite to quickly adapt to changing environments. It is plausible that reactivation of mature cysts needs a robust energy supply and catabolism of amylopectin is a critical contributor, which may be more difficult to achieve through exogenous glucose in bradyzoites when compared to tachyzoites.

Previous work has demonstrated that CDPK2 inactivation caused over-accumulation of amylopectin in tachyzoites and bradyzoites [20]. The Δcdpk2 mutant displays a reduced virulence and does not form cysts in vivo, although it is not clear whether abnormal amylopectin accumulation is directly responsible for these defects. Our results showed that SS deletion abolished amylopectin accumulation and restored the virulence and cyst formation defects in the Δcdpk2 mutant, revealing that those deficiencies are indeed caused by the overly accumulated starch. Due to a modest attenuation of virulence and lack of cyst formation, the mutants lacking CDPK2 were proposed as a live vaccine candidate [33]. However, our work raises safety concerns to this vaccine candidate, as the Δcdpk2 mutants can readily regain normal virulence and cyst formation capacity by inactivating genes like SS involved in SS.

Ethics. All animal experiments were approved by the Scientific Ethics Committee of Huazhong Agricultural University (permit no. HZAU-2018-034).

Data accessibility. This article has no additional data.

Authors’ contributions. B.S. designed the study, C.L., X.Y., J.Y. and L.H. performed the experiments and analysed the data, C.L. and B.S. wrote the paper, Y.Z. and J.Z. provided materials and analysed the data.

Competing interests. We declare we have no competing interests.

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