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Differential contribution of two organelles of endosymbiotic origin to iron-sulfur cluster synthesis in Toxoplasma

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Abstract

Iron-sulfur (Fe-S) clusters are one of the most ancient and ubiquitous prosthetic groups, and they are required by a variety of proteins involved in important metabolic processes. Apicomplexan parasites have inherited different plastidic and mitochondrial Fe-S clusters biosynthesis pathways through endosymbiosis. We have investigated the relative contributions of these pathways to the fitness of *Toxoplasma gondii*, an apicomplexan parasite causing disease in humans, by generating specific mutants. Phenotypic analysis and quantitative proteomics allowed us to highlight striking differences in these mutants. Both Fe-S cluster synthesis pathways are necessary for optimal parasite growth in vitro, but their disruption leads to markedly different fates: impairment of the plastidic pathway leads to a loss of the organelle and to parasite death, while disruption of the mitochondrial pathway trigger differentiation into a stress resistance stage. This highlights that otherwise similar biochemical pathways hosted by different sub-cellular compartments can have very different contributions to the biology of the parasites, which is something to consider when exploring novel strategies for therapeutic intervention.

Keywords: iron sulfur cluster, Toxoplasma, differentiation, bradyzoite, apicoplast, mitochondrion

Introduction

Endosymbiotic events were crucial in the evolutionary timeline of eukaryotic cells. Mitochondria and plastids evolved from free-living prokaryotes that were taken up by early eukaryotic ancestors and transformed into permanent subcellular compartments that have become essential for harnessing energy or synthesizing essential metabolites in present-day eukaryotes (1). As semiautonomous organelles, they contain a small genome, but during the course of evolution a considerable part of their genes have been transferred to the cell nucleus. Yet, they rely largely on nuclear factors for their maintenance and expression. Both organelles are involved in critically important biochemical processes. Mitochondria, which are found in most eukaryotic organisms, are mostly known as the powerhouses of the cell, owing to their ability to produce ATP through respiration. Importantly, they are also involved in several other metabolic pathways (2), including the synthesis of heme groups, steroids, amino acids, and iron-sulphur (Fe-S) clusters. Moreover, they have important cellular functions in regulating redox and calcium homeostasis. Similarly, plastids that are found in plants, algae and some other eukaryotic organisms host a diverse array of pathways that contribute greatly

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to the cellular metabolism (3). While often identified mainly as compartments where photosynthesis occurs, plastids host many more metabolic pathways. For example, they are involved in the assimilation of nitrogen and sulfur, as well as the synthesis of carbohydrates, amino acids, fatty acids and specific lipids, hormone precursors, and also Fe-S clusters. The best-characterized plastid is arguably the plant cell chloroplast, but not all plastids have photosynthetic function, and in higher plants they are in fact a diverse group of organelles that share basal metabolic pathways, but also have specific physiological roles (4). As documented in plants, although mitochondria and plastids are highly compartmentalized (5), they have metabolic exchanges and cooperate in the context of several important metabolic pathways (6).

The phylum Apicomplexa comprises a large number of single-celled protozoan parasites responsible for cause serious disease in animals and humans. For example, this phylum includes parasites of the genus *Plasmodium* that are responsible for the deadly malaria, and *Toxoplasma gondii* a ubiquitous parasite that can lead to a severe pathology in immunocompromised individuals. Apicomplexan parasites evolved from a photosynthetic ancestor and many of them still retain a plastid (7, 8). This plastid, named the apicoplast, originated from a secondary endosymbiotic event: the eukaryotic ancestor of Apicomplexa engulfed and retained a eukaryotic alga that was already containing a plastid obtained by primary endosymbiosis of a cyanobacterium-like prokaryote (9, 10). It has lost its photosynthetic properties as the ancestors of Apicomplexa switched to an intracellular parasitic lifestyle (11). The apicoplast nevertheless still hosts four main metabolic pathways (12, 13): a 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-d-xylulose 5-phosphate (MEP/DOXP) pathway for the synthesis of isoprenoid precursors, a type II fatty acid synthesis pathway (FASII), part of the heme synthesis pathway, and a Fe-S cluster synthesis pathway. As the apicoplast is involved in these vital biological processes for the parasite, and as they markedly differ from those of the host (because of their algal origin), that makes it a valuable potential drug target. Apicomplexan parasites also generally contain a single tubular mitochondrion, although its aspect may vary during parasite development (14, 15). The organelle is an important contributor to the parasites metabolic needs (16). It classically hosts tricarboxylic acid (TCA) cycle reactions, which are the main source of electrons that feeds the mitochondrial electron transport chain (ETC) and generate a proton gradient used for ATP production. It also contains additional metabolic pathways, like a Fe-S cluster synthesis pathway and part of the heme synthesis pathway operating in collaboration with the apicoplast. The latter reflects obvious functional links between the organelles and potential metabolic interactions, which is also illustrated by their physical connection during parasite development (17, 18). Because of their endosymbiotic origin, these organelles offer possibilities for intervention against Apicomplexa and are currently the target of treatments (19). For instance, as their protein synthesis machinery is bacterial in nature, both may therefore be a target of bacterial translation inhibitors such as azithromycin, spiramycin or clindamycin (20). However, current evidence suggests that the apicoplast is the primary target of these drugs. The mitochondrion, on the other hand, is an important drug target through the ETC it harbours, which is inhibited by drugs such as atovaquone (21).

Fe-S clusters are simple and ubiquitous cofactors involved in a great variety of cellular processes. As their name implies, they are composed of iron and inorganic sulfur whose chemical properties confer key structural or electron transfer features to proteins in all kingdoms of life. They are important to the activities of numerous proteins that play essential roles to sustain fundamental life processes including, in addition to electron transfer and exchange, iron storage, protein folding, oxygen/nitrogen stress sensing, and gene regulation (22). The synthesis of Fe-S clusters and their insertion into apoproteins requires complex machineries and several distinct pathways have been

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identified in bacteria for synthesizing these ancient cofactors (23). They include the ISC (iron sulfur cluster) pathway for general Fe–S cluster assembly (24), and the SUF (sulfur formation) pathway (25) that is potentially activated in oxidative stress conditions (26). Eukaryotes have inherited machineries for synthesizing Fe-S cluster through their endosymbionts (27). As a result, organisms with both mitochondria and plastids, like higher plants, use the ISC pathway for assembling Fe-S clusters in the mitochondria and the SUF pathway for Fe-S clusters in the plastids (28). Additional protein components that constitute a cytosolic Fe-S cluster assembly machinery (CIA) have also been identified: this pathway is important for the generation of cytosolic, but also of nuclear Fe-S proteins, and is highly dependent on the ISC mitochondrial pathway for providing a sulfur-containing precursor (29).

Like in plants and algae, apicoplast-containing Apicomplexa seem to harbour the three ISC, SUF and CIA Fe-S cluster synthesis pathways. Although the CIA pathway was recently shown to be important for *Toxoplasma* fitness (30), investigations in apicomplexan parasites have been so far almost exclusively focused on the apicoplast-located SUF pathway (31–35) and mostly in *Plasmodium* species. The SUF pathway was shown to be essential for the viability of malaria parasites during both the erythrocytic and sexual stages of development and has thus been recognized as a putative avenue for discovering new antiparasitic drug targets (reviewed in (36)). Contrarily to the ISC pathway, which is also present the mammalian hosts of apicomplexan parasites, the SUF pathway may indeed yield interesting specificities that may be leveraged for therapeutic intervention. However, very little is known about Fe-S clusters synthesis in other apicomplexan parasites, including *T. gondii*. For instance, out of the four known metabolic pathways hosted by the apicoplast, Fe-S synthesis was the only one remaining to be functionally investigated in *T. gondii*, while the others were all shown to be essential for the tachyzoite stage of the parasite (a fast replicating developmental stage responsible for the symptoms of the disease) (37–40). Here, we present the characterization of two *T. gondii* mutants we generated to specifically impact the plastidic and mitochondrial SUF and ISC pathways, respectively. Our goal was to assess the relative contributions of these compartmentalized pathways to the parasite development and fitness.

Results

TgSufS and TgIscU are functional homologs of components of the plastidic and mitochondrial iron sulfur cluster synthesis pathways

Fe-S cluster biosynthesis pathways in the mitochondrion and the plastid follow a similar general pattern: cysteine desulfurases (IscS, SufS) produce sulfur from L-cysteine, scaffold proteins (IscU, SufB/C/D) provide a molecular platform allowing iron and sulfur to meet and form a cluster, and finally carrier proteins (like IscA or SufA) deliver the cluster to target apoproteins (28). The cytosolic CIA pathway, which is responsible for the de novo formation of Fe-S clusters to be incorporated in cytosolic and nuclear proteins, is dependent on the ISC pathway, as its first step requires the import of a yet unknown sulfur-containing precursor that is translocated to the cytosol from the mitochondrion (29). To get a general overview of the predicted components for the Fe-S cluster machinery in *T. gondii*, we conducted homology searches in the ToxoDB.org database (41), using well-characterized proteins from plants (*Arabidopsis thaliana*) belonging to the SUF, ISC and CIA pathways (Table S1). Data from global mapping of protein subcellular location by HyperLOPIT spatial proteomics (42) was in general in good accordance with the expected localization of the homologs (with the noticeable exception of members of the NBP35/HCF101 ATP-binding proteins). Overall, our search revealed that *T. gondii* appeared to have a good conservation of all the main components of

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the three ISC, SUF and CIA Fe-S synthesis pathways (Table S1, Figure 1A). Additional information available on ToxoDB.org such as scores from a CRISPR/Cas9-based genome-wide screening (43), highlighted that most components of the three pathways are important for parasite fitness. This suggests several apoproteins localizing to the endosymbiotic organelles, but also the cytosol/nucleus, are essential for the optimal growth of tachyzoites.

In order to verify this, we decided to generate mutants of the apicoplast-localized SUF pathway and of the mitochondrion-localized ISC pathway in *T. gondii* tachyzoites. To this end, we targeted the SufS and IscU homologs, which are both central (and presumably essential) to their respective pathways (Figure 1A). We first sought to verify TgSufS (TGGT1_216170) and TgIscU (TGGT1_237560) were real functional homologs by performing complementation assays of bacterial mutants. Expression of the predicted functional domains of TgSufS and TgIscU in the respective *Escherichia coli* mutants improved bacterial growth in the presence of an iron chelator or not (Figure 1B). This suggests TgSufS and TgIscU, in addition to a good sequence homology with their bacterial homologues (Figure S1), have a conserved function.

We next determined the sub-cellular localizations of TgSufS and TgIscU by epitope tagging of the native proteins. This was achieved in the TATi ΔKu80 cell line, which favors homologous recombination and would allow transactivation of a Tet operator-modified promoter we would later use for generating a conditional mutant in this background (44–46). A sequence coding for a C-terminal triple hemagglutinin (HA) epitope tag was inserted at the endogenous *TgSufS* or *TgIscU* locus by homologous recombination (Figure S2). Using the anti-HA antibody, by immunoblot we detected two products for each protein (Figure 2A, B), likely corresponding to their immature and mature forms (ie after cleavage of the transit peptide upon import into the organelle). Accordingly, the analysis of TgSufS and TgIscU sequences with several subcellular localization and N-terminal sorting signals site predictors confirmed they likely contained sequences for plastidic and mitochondrial targeting (47), respectively, although no consensus position of the exact cleavage sites could be determined. Immunofluorescence assay (IFA) in *T. gondii* tachyzoites confirmed HA-tagged TgSufS and TgIscU co-localized with markers of the apicoplast and the mitochondrion, respectively (Figure 2C, D).

SufS is a cysteine desulfurase whose activity is enhanced by an interaction with the SufE protein (48). Similarly to plants that express several SufE homologues (49), there are two putative SufE-like proteins in *T. gondii* (Table S1), one of which was already predicted to reside in the apicoplast by hyperLOPIT (TgSufE1, TGGT1_239320). We generated a cell line expressing an HA-tagged version of the other, TgSufE2 (TGGT1_277010, Figure S3A, B, C), whose localization was previously unknown. Like for TgSufS, several programs predicted a plastidic transit peptide, which was confirmed by immunoblot analysis (detecting TgSufE2 immature and mature forms, Figure S3D). IFA showed TgSufE2 co-localized with an apicoplast marker (Figure S3E). This further confirms that the initial steps of Fe-S cluster biogenesis in the apicoplast are likely functionally-conserved.

Disruption of either the plastidic or the mitochondrial Fe-S cluster pathway has a profound impact on parasite growth

In order to get insights into plastidic and mitochondrial Fe-S biogenesis, we generated conditional mutant cell lines in the TgSufS-HA or TgIscU-HA-expressing TATi ΔKu80 background (46). Replacement of the endogenous promoters by an inducible-Tet07*SAG4* promoter, through a single homologous recombination at the loci of interest (Figure S4), yielded conditional TgSufS and TgIscU conditional knock-down cell lines (cKD TgSufS-HA and cKD TgIscU-HA, respectively). In these cell

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lines, the addition of anhydrotetracycline (ATc) can repress transcription through a Tet-Off system (50). For each cKD cell line several transgenic clones were obtained and found to behave similarly in the initial phenotypic assays we performed, so only one was further analyzed. Transgenic parasites were grown for various periods of time in presence of ATc, and protein down-regulation was evaluated. Immunoblot and IFA analyses of cKD TgSufS-HA and cKD TgIscU-HA parasites showed that the addition of ATc efficiently down-regulated the expression of TgSufS (Figure 3A, C) and TgIscU (Figure 3B, D), and most of the proteins were undetectable after two days of incubation.

We also generated complemented cell lines expressing constitutively an additional copy of *TgSufS* and *TgIscU* from the *uracil phosphoribosyltransferase* (*UPRT*) locus from a *tubulin* promoter in their respective conditional mutant backgrounds (Figure S5A, B). We confirmed by semi-quantitative RT-PCR (Figure S5C) that the transcription of *TgSufS* and *TgIscU* qenes was effectively repressed in the cKD cell lines upon addition of ATc, whereas the corresponding complemented cell lines exhibited a high transcription level regardless of ATc addition (due to the expression from the strong *tubulin* promoter).

We next evaluated the consequences of TgSufS and TgIscU depletion on *T. gondii* growth in vitro. First, to assess the impact on the parasite lytic cycle, the capacity of the mutants and complemented parasites to produce lysis plaques was analyzed on a host cells monolayer in absence or continuous presence of ATc for 7 days (Figure 4A, B). Depletion of both proteins completely prevented plaque formation, which was restored in the complemented cell lines. To assess whether this defect in the lytic cycle is due to a replication problem, all cell lines were preincubated in ATc for 48 hours and released mechanically, before infecting new host cells and growing them for an additional 24 hours in ATc prior to parasite counting. We noted that incubation with ATc led to an accumulation of vacuoles with fewer parasites, yet that was not the case in the complemented cell lines (Figure 4C, D). Overall, these data show that either TgSufS or TgIscU depletion impacts parasite growth.

Then, we sought to assess if the viability of the mutant parasites was irreversibly affected. We thus performed a similar experiment, but at the end of the 7-day incubation, we washed out the ATc, incubated the parasites for an extra 4 days in the absence of the drug and evaluated plaque formation (Figure 4E). In these conditions, while cKD TgSufS-HA parasites displayed very few and very small plaques suggesting their viability was irreversibly impacted, cKD TgIscU-HA parasites showed considerable plaque numbers. However, comparing plaque number between the 7-day and 4-day washout conditions in wells were the same initial dose of cKD TgIscU-HA parasites was added, we could determine that only 28%±2% of plaques were formed after ATc removal (n=3 independent biological replicates), suggesting some mortality.

We performed IFAs to assess possible morphological defects that may explain the impaired growths of cKD TgSufS-HA and cKD TgIscU-HA parasites. We stained the apicoplast and mitochondrion of parasites kept in the continuous presence of ATc for several days. cKD TgSufS-HA parasites managed to grow and egress after three days and were seeded onto new host cells, where there were kept for two more days in the presence of ATc. During this second phase of intracellular development, and in accordance with the replication assays (Figure 4C), growth was slowed down considerably. Strikingly, while the mitochondrial network seemed normal, we noticed a progressive loss of the apicoplast (Figure 5A), which was quantified (Figure 5B). The growth kinetics we observed for this mutant are consistent with the “delayed death” effect observed in apicoplast-defective parasites (8, 51, 52). On the other hand, we were able to grow cKD TgIscU-HA parasites for five days of continuous culture: they developed large vacuoles and showed little sign of egress from the host cells (Figure 5C). Both the mitochondrion and the apicoplast appeared otherwise normal morphologically. These large vacuoles could reflect a default in the egress of parasites during the lytic cycle (53). We thus

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224 performed an egress assay on cKD TgIscU-HA parasites that were kept for up to five days in the
225 presence of ATc, and they were able to egress normally upon addition of a calcium ionophore (Figure
226 5D). These large vacuoles are also reminiscent of cyst-like structures (54), so alternatively this may
227 reflect spontaneous stage conversion. Cysts are intracellular structures that contain the slow-growing
228 form of *T. gondii*, called the bradyzoite stage (which is responsible for the chronic phase of the
229 disease), and they may appear even during in vitro growth in particular stress conditions (55).

230 In any case, our data show that interfering with the plastidial and mitochondrial Fe-S protein
231 pathways both had important consequences on parasite growth, but had a markedly different impact
232 at a cellular level.

233

234 **Use of label-free quantitative proteomics to identify pathways affected by TgSufS or TgIscU**
235 **depletion**

236 There is a wide variety of eukaryotic cellular processes that are depending on Fe-S cluster proteins.
237 To get an overview of the potential *T. gondii* Fe-S proteome, we used a computational tool able to
238 predict metal-binding sites in protein sequences (56) and performed subsequent manual curation to
239 refine the annotation. We identified 64 proteins encompassing various cellular functions or
240 metabolic pathways that included, beyond the Fe-S synthesis machinery itself, several DNA and RNA
241 polymerases, proteins involved in redox control and electron transfer and radical S-
242 adenosylmethionine (SAM) enzymes involved in methylation and methylthiolation (Table S2).
243 HyperLOPIT data or manual curation helped us assign a putative localization for these canditates. A
244 considerable proportion (19%) of these were predicted to localize to the nucleus, where many
245 eukaryotic Fe-S proteins are known to be involved in DNA replication and repair (57). Yet, strikingly,
246 most of the predicted Fe-S proteins likely localize to the endosymbiotic organelles. Several (19%) are
247 predicted to be apicoplast-resident proteins, including radical SAM enzymes lipoate synthase (LipA)
248 (58) and the MiaB tRNA modification enzyme (59), as well as the IspG and IspH oxidoreductases of
249 the MEP pathway (60). Finally, for the most part (43%) candidate Fe-S proteins were predicted to be
250 mitochondrial, with noticeably several important proteins of the respiratory chain (Fe-S subunit of
251 the succinate dehydrogenase complex, Rieske protein and TgApiCox13) (61–63), but also enzymes
252 involved in other metabolic pathways such as heme or molybdopterin synthesis. CRISPR/Cas9 fitness
253 scores (43) confirmed many of these putative Fe-S proteins likely support essential functions for
254 parasite growth.

255 We sought to confirm these results experimentally. Thus, in order to uncover the pathways primarily
256 affected by the depletion of TgIscU and TgSufS, and to identify potential Fe-S protein targets, we
257 conducted global label free quantitative proteomic analyses. Like most plastidic or mitochondrial
258 proteins, candidate Fe-S acceptors residing in these organelles are nuclear-encoded and thus need to
259 be imported after translation and have to be unfolded to reach the stroma of the organelle. This not
260 only implies the addition of the Fe-S cofactor should happen locally in the organelle, but also that this
261 may have a role in proper folding of these proteins. We thus assumed that disrupting a specific
262 pathway may have a direct effect on the stability and expression levels of local Fe-S proteins. Cellular
263 downstream pathways or functions may also be affected, while other pathways may be upregulated
264 in compensation. Parasites were treated for two days with ATc (TgIscU-HA) or three days (cKD
265 TgSufS-HA, as it takes slightly longer to be depleted, Figure 3A) prior to a global proteomic analysis
266 comparing protein expression with the ATc-treated TATi ΔKu80 control. For each mutant, we
267 selected candidates with a log2(fold change) ≤-0.55 or ≥0.55 (corresponding to a ~1.47 fold change in
268 decreased or increased expression) and a p-value <0.05 (ANOVA, *n*=4 biological replicates) (Tables S3

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and S4, Figure 6A). To get a more exhaustive overview of proteins whose amounts varied drastically, we completed this dataset by selecting some candidates that were consistently and specifically absent from the mutant cell lines or only expressed in these (Tables S3 and S4).

Overall, depletion of TgIscU led to a higher variability in protein expression and while the pattern of expression was essentially specific for the respective mutants, a number of shared variant proteins were found (Figure 6B, Table S5). For instance, common lower expressed candidates include a SAM synthase, possibly reflecting a general perturbation of SAM biosynthesis upon loss of function of Fe-S-containing radical SAM enzymes (64). Using dedicated expression data (65, 66) available on ToxoDB.org we realized that, strikingly, many of the common variant proteins were stage-specific proteins (Table S5). For instance, the protein whose expression went down the most is SAG-related sequence (SRS) 19F. The SRS family contains GPI-anchored surface antigens related to SAG1, the first characterized *T. gondii* surface antigen, and whose expression is largely stage-specific (67). This protein, SRS19F is expressed in bradyzoites, but may be most highly expressed in stages present in the definitive host (66, 68). Conversely, SRS44, also known as CST1 and one of the earliest marker of stage conversion to bradyzoites (69), was upregulated in both mutants. Several other bradyzoite proteins whose expression increased included Ank1, a tetratricopeptide-repeat protein highly upregulated in the cyst-stages but not necessary for stage conversion (70), aspartyl protease ASP1, an α-galactosidase, as well as several dense granule proteins (GRA). Dense granules are specialized organelles that secrete GRA proteins that are known to participate in nutrient acquisition, immune evasion, and host cell-cycle manipulation. Many GRA have been characterized in the tachyzoite stage, but several stage-specific and expressed in bradyzoites (71). It should be noted that bradyzoite-specific proteins were generally much strongly expressed upon TgIscU depletion than TgSufS depletion. Nevertheless, altogether these results show that altering either the plastidic or the mitochondrial Fe-S cluster synthesis pathway led to an initial activation of the expression of some markers of the bradyzoite stage, whose involvement in the stress-mediated response is well documented (55).

Depletion of TgSufS has an impact on the apicoplast, but also beyond the organelle

We next focused on proteins that varied upon depletion of TgSufS (Table S3). Using the hyperLOPIT data available on ToxoDB.org, we assessed the putative localization of the candidates (Figure 7A) and we also defined putative functional classes based on manual curation (Figure 7B). Surprisingly, few apicoplast proteins were impacted. This could reflect a limited impact on apicoplast Fe-S apoproteins, but this is in contradiction with the strong and specific effect we see on the organelle in the absence of TgSufS (Figure 5A, B). There might also be a bias due to an overall low protein abundance: less than half of the apicoplast candidates of the predicted Fe-S proteome (Table S2) were robustly detected even in the control for instance, including our target protein SufS. Finally, of course it is possible that depletion of Fe-S clusters, while impacting the functionality of target proteins, did not have a considerable effect on their abundance. We sought to verify this for apicoplast stroma-localized LipA, a well-established and evolutionarily-conserved Fe-S cluster protein, which was found to be only marginally less expressed in our analysis (Table S3). LipA is responsible for the lipoylation of a single apicoplast target protein, the E2 subunit of the pyruvate dehydrogenase (PDH) (37). Using an anti-lipoic acid antibody on cKD TgSufS-HA protein extracts, we could already see a marked decrease in lipoylated PDH-E2 after only one day of ATc incubation (Figure 7C). This was not due to a general demise of the apicoplast as it considerably earlier than the observed loss of the organelle (Figure 5A, B), and levels of the CPN60 apicoplast marker were clearly

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not as markedly impacted (Figure 7C). This finding confirmed apicoplast Fe-S-dependent activities are specifically affected in our mutant, before observing the general demise and loss of the organelle.

Other potential apicoplast Fe-S cluster-containing proteins include IspG and IspH, key enzymes of the MEP isoprenoid synthesis pathway (60). Again, these proteins were only found marginally less expressed in our quantitative analysis, yet our proteomics dataset provided indirect clues that their function may be impacted. Isoprenoids precursors can be used as lipophilic groups to modify proteins, but may also be incorporated into lipids like ubiquinone, which is an important polyprenylated cofactor of the mitochondrial respiratory chain. Quite strikingly, a single predicted mitochondrial candidate was significantly less expressed upon TgSufS depletion and is homolog of the UbiE/COQ5 methyltransferase, which is involved in ubiquinone synthesis (72). Isoprenoids are also important for dolichol-derived protein glycosylation and glycosylphosphatidylinositol (GPI)-anchor biosynthesis. That may account for effects of TgSufS depletion on specific proteins. For instance, the three potentially rhoptry-localized candidates significantly less expressed (Table S3) are predicted to be GPI-anchored and/or glycosylated. Overall, this might be an indication that TgSufS depletion impacts isoprenoid synthesis in the apicoplast, which in turn would impact other metabolic pathways.

There were additional indications that TgSufS depletion has consequences beyond apicoplast metabolism, as we noticed clear variations in other proteins residing in other subcellular compartments. For instance, changes in expression of stage-specific GRA and SRS proteins reflecting, as mentioned before, a possible initiation of stage conversion to bradyzoites. Interestingly, the higher expression of Golgi apparatus/plasma membrane transporters or endoplasmic reticulum (ER)-located lipid-related enzymes suggest some sort of metabolic adaptation occurs upon depletion of TgSufS (Figure 7A, B). The apicoplast and the ER cooperate for fatty acid (FA) and phospholipid (PL) synthesis (73). The apicoplast generates short FA chains through the FASII system, but also lysophosphatidic acid (LPA) as a PL precursor (74), and FA chains can then be further modified by ER-localized enzymes that include elongases. Yet, these the ER-localized PL-synthesis machinery can also use FA scavenged from the host (75). The increased expression of ER-localized lipid-related enzymes may thus reflect an increased synthesis, potentially from exogenous lipid precursors, in compensation from a defect in the apicoplast-localized machinery. Overall, this suggests impacting the Fe-S cluster synthesis pathway in the apicoplast had important metabolic consequences beyond the organelle itself.

Depletion of TgIscU impacts the respiratory capacity of the mitochondrion and leads to stage conversion

We also analyzed the proteins whose abundance changed upon TgIscU depletion (Table S4). Again, we used hyperLOPIT data to determine the localization of variant proteins (Figure 8A) and we also inferred their potential function from GO terms or manual curation (Figure 8B). Depletion of TgIscU had a notable impact locally, as numerous mitochondrial proteins were found in lower abundance. Remarkably, most of these proteins were identified as members of the mitochondrial respiratory chain. This ETC comprises five complexes, in which several Fe-S proteins have important function. As mentioned earlier, they include the iron-sulfur subunit of the succinate dehydrogenase complex (complex II), the Rieske protein (part of complex III, with cytochrome *b* and *c*1) and TgApiCox13 (part of complex IV, the cytochrome *c* oxidase) (61–63). Not only these three Fe-S cluster proteins were found to be less expressed upon TgIscU depletion, but most components of the complexes III and IV (including recently characterized parasite-specific subunits (62, 63)) were also significantly less abundant (Table S4). This suggested the mitochondrial membrane potential and consequently the

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respiratory capacity of the mitochondrion were likely altered in the absence of a functional mitochondrial Fe-S cluster synthesis pathway. To verify this, we performed flow cytometry quantification using JC-1, a monomeric green fluorescent carbocyanine dye that accumulates as a red fluorescent aggregates in mitochondria depending on their membrane potential (Figure 9A). Depletion of TgIscU led to a marked decrease of the parasite population displaying a strong red signal (Figure 9B). The effect was maximal after two days of ATc treatment and not further increased by a four-day treatment, which is consistent with the quantitative proteomics data already showing strong impact on proteins from complexes II, III and IV after only two days of ATc treatment. Concomitantly to the lesser expression of mitochondrial respiratory chain subunits, the proteomics analysis revealed TgIscU depletion induced a significant increase in cytosolic enzymes involved in glycolysis, as well as its branching off pentose phosphate pathway (Figure 8A, B, Table S4). The upregulation of glycolytic enzymes potentially reflects a metabolic compensation for mitochondrial defects in energy production due to the impairment of the respiratory chain. Other proteins whose abundance was markedly decreased were predicted to cytoplasmic or nuclear, which is perhaps unsurprising as the cytosolic CIA Fe-S cluster assembly pathway is supposedly dependent from the SUF pathway (29). The changes in abundance of several RNA-binding proteins involved in mRNA half-life or transcription/translation regulation may also reflect adaptation to a stress.

Indeed, another feature highlighted by the quantitative proteomics analysis of the TgIscU mutant is the change in the expression of stage-specific proteins (Table S4). The expression of several bradyzoite-specific including GRAs and proteins of the SRS family, was strongly increased. At the same time, some tachyzoite-specific SRS and GRA proteins were found to be less expressed. This was supporting the idea that intracellularly developing parasites lacking TgIscU may convert into bona fide cyst-contained bradyzoites, as suggested by our initial morphological observations (Figure 5C). To verify this, we used a lectin from the plant *Dolichos biflorus*, which recognizes the SRS44/CST1 glycoprotein that is exported to the wall of differentiating cysts (69). We could see that during continuous growth of cKD TgIscU-HA parasites in the presence of ATc, there was an increasing number of DBL-positive structures (Figure 10A). This was quantified during the first 48 hours of intracellular development (Figure 10B) and, interestingly, was shown to mimic the differentiation induced by nitric oxide, a known factor of stage conversion (76), and a potent damaging agent of Fe-S clusters (77). We combined RNAseq expression data for tachyzoite and bradyzoite stages (66) to establish a hierarchical clustering of the SRS proteins detected in our quantitative proteomics experiments for the two mutants (Figure 10C). This clearly confirmed a strong increase in the expression of bradyzoite-specific SRS in the TgIscU mutant. As mentioned earlier, some were also upregulated in the TgSufS mutant but in much lesser proportions. The strongest increase in bradyzoite-specific SRS expression upon TgSufS depletion was for SRS44/CST1, which happens to be the protein DBL preferentially binds to (69). However, contrarily to the TgIscU mutant, labelling experiments did not indicate any detectable increase in DBL recruitment in the TgSufS mutant (Figure 10B), confirming that impairing the plastidic Fe-S center synthesis pathway does not trigger full stage conversion in this cell line. Stage conversion is a progressive process that happens over the course of several days, as it involves the expression of distinct transcriptomes and proteomes (55). Markers for specific steps of in vitro cyst formation had been previously described (78), so we have used several of these to check the kinetics of stage conversion in the TgIscU-depleted parasites. We kept the cKD TgIscU-HA parasites for up to 20 days in the presence of ATc and tested for the presence of SAG1 (tachyzoite maker), DBL (early bradyzoite marker), P18/SAG4 (intermediate bradyzoite marker) and P21 (late bradyzoite marker) (Figure 10D). After 7 days of ATc treatment, the DBL-positive cyst contained parasites were still expressing SAG1 and not yet SAG4, whereas after 20 days parasites with SAG4 labelling were found, but there was still a residual SAG1 expression; expression of late

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marker P21 was, however, never detected. This suggests stage conversion of these parasites progresses beyond the appearance of early cyst wall markers, but not only it does so with slow kinetics, but it seems incomplete. In fact, observation of DBL-positive cysts showed a marked decrease in their mean size between the 7 and 20 days timepoints (Figure 10D). This suggests incomplete conversion may be leading to subsequent reactivation/reinvasion events. There is also possibly a lack of fitness in the long term for the TgIscU-depleted converting parasites, which would be in accordance with our plaque assays that showed not all mutant parasites were able to grow back upon ATc removal.

Discussion

Because of their origin and metabolic importance, the two apicomplexan endosymbiotic organelles have gathered considerable interest as potential drug targets (79, 80). It may be obvious as for example the plastid hosts several metabolic pathways which are not present in the mammalian hosts of these parasites. Yet, even for conserved housekeeping functions or, in the case of the mitochondrion early phylogenetic divergence, may still provide enough molecular differences to allow selective chemical inhibition. In fact, several drugs used for prophylactic or curative treatments against Apicomplexa-caused diseases are already targeting these organelles. They are essentially impacting the organellar protein synthesis by acting on the translation machinery (81), although the mitochondrial respiratory chain inhibitor atovaquone is also used to treat malaria and toxoplasmosis (82). One main difference when targeting *Plasmodium* and *Toxoplasma* by drugs is that the latter easily converts into the encysted bradyzoite resistance form. It has been known for some time that treatment of tachyzoites with mitochondrial inhibitors triggers stage conversion (76, 83, 84). This may be efficient to counteract the acute phase of toxoplasmosis, but at the same time may favour persistence of the parasites in the host.

Here we characterized pathways which are very similar biochemically, but are located into two distinct endosymbiotic organelles and whose inactivation has drastically different effects on the parasites. Fe-S clusters are ancient, ubiquitous and fundamental to many cellular functions, but their synthesis by distinct biosynthetic pathways was inherited by specific endosymbiotic organelles through distinct bacterial ancestors, and have thus specialized into adding these cofactors to different client proteins (27). A key function of Fe-S clusters, owing to their mid-range redox potential, is electron transfer and redox reactions, mainly as components the respiratory and photosynthetic electron transfer chains. They also have important functions in stabilizing proteins, redox sensing, or catalysis through SAM enzymes. Several of these are not retained in Apicomplexa, whose plastid has lost its photosynthetic ability for example. Nevertheless, our prediction of the *T. gondii* Fe-S proteins repertoire suggests many key functions associated with the apicoplast or the mitochondrion are likely to be affected by a perturbation of Fe-S assembly (Table S2).

For the apicoplast, these include lipoic acid or isoprenoid synthesis. Inactivation of the apicoplast-located TgSufS had a marked effect on the organelle itself, as it led ultimately to a loss of the apicoplast, which is consistent with the phenotype observed when disrupting the Suf pathway in *Plasmodium* (31). Isoprenoid synthesis is vital for *T. gondii* tachyzoites (38), and it has implication beyond the apicoplast, as prenylated proteins or isoprenoid precursors are involved in more general cellular processes including intracellular trafficking or mitochondrial respiration (85). Impairing isoprenoid synthesis does not, however, necessarily lead to a loss of the organelle (31). There may thus be another explanation for this phenotype. Interestingly, we could show that perturbing the Suf pathway, which is supposedly important for Fe-S-containing enzyme LipA, impacts the lipoylation of

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E2 subunit of the apicoplast-located PDH (Figure 7C). The PDH complex catalyzes the production of acetyl-CoA, which is the first step of the FASII system, and perturbation of either the PDH or other steps of the FASII system lead to a loss of the organelle and severely impairs fitness of the parasites (38, 86). Our quantitative proteomic analysis shows potential compensatory mechanisms may be used by the parasites in response this early perturbation of the apicoplast lipid metabolism that precedes organelle loss. Tachyzoites are indeed known to be able to use exogenous lipid sources to adapt metabolically (86, 87), and interestingly upon depletion of TgSufS we observed a pattern of overexpression for ER-located enzymes involved in the synthesis of several phospholipids and ceramides (Table S3). These lipids are usually synthesized in the ER from apicoplast-synthesized precursors, but this may clearly indicate a compensatory mechanism that would make use of precursors scavenged form the host instead. In spite of this, it seems the alteration of the Suf pathway in *T. gondii* has such a profound impact on the apicoplast itself, that is causes a typical "delayed death" phenotype that ultimately leads to the irreversible demise of the parasites (Figure 4).

For the mitochondrion, important pathways potentially involving Fe-S proteins include the respiratory ETC, the TCA cycle, as well as molybdenum and heme synthesis (Table S2). Accordingly, perhaps the most obvious consequence of disrupting the ISC pathway was the profound impact on the mitochondrial respiratory capacity, as evidenced experimentally by measuring the mitochondrial membrane potential (Figure 9), and supported by proteomic analyses showing a clear drop in expression of many respiratory complex proteins (Table S4). Although the mitochondrion, through the TCA cycle and the respiratory chain/oxidative phosphorylation, contributes to energy production in tachyzoites (88), the glycolytic flux is also believed to be a major source of carbon and energy for these parasites (89). Thus, rather coherently, as highlighted by our quantitative proteomic analysis, disruption of the ISC pathway led to the overexpression of glycolytic enzymes concurrently with the lower expression of mitochondrial ETC components. The overexpression of enzymes of the pentose phosphate pathway, which is branching off from glycolysis and is providing redox equivalents and precursors for nucleotide and amino acid biosynthesis, is also potentially indicative of a higher use of glucose in these conditions. The metabolic changes encountered by SUF-deficient parasites do not cause their rapid demise, as they are able to initiate conversion to the bradyzoite stage, which has been suggested to rely essentially on glycolysis for energy production anyway (90).

The transition from tachyzoite to bradyzoite is known to involve a considerable change in gene expression (65, 66), and it takes several days of in vitro differentiation-inducing conditions to obtain mature cysts (91, 92). TgIscU-depleted parasites rapidly displayed a high expression of bradyzoite-specific surface antigens and GRA markers (Table S4, Figure 10), and as they developed they were included in structures with typical cyst-like morphology (Figure 5, Figure 10). However, using specific antibodies against early or late bradyzoite markers, we could see that even when depleting TgIscU for an extended time period, the differentiating parasites never appeared to reach fully mature bradyzoite stage (Figure 10). One of the reason is that our mutants were generated in a type I *T. gondii* strain, which is associated with acute toxoplasmosis in the mouse model (93) and typically does not form cysts: type I tachyzoites may upregulate specific bradyzoite genes and, according to some reports, produce bradyzoite-specific proteins or cyst wall components, but are largely incapable of forming mature bradyzoite cysts (94). A second explanation is that these parasites may not be viable long enough to fully differentiate. For instance, although we found the impact of TgIscU depletion on the lytic cycle was partly reversible, a large proportion of the parasites was not able to recover after 7 days of ATc treatment. This may not be solely due to the alteration of the mitochondrial metabolism, as the inactivation of the ISC pathway likely has consequences on other important cellular housekeeping functions. In other eukaryotes, the SUF pathway provides a yet

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unknown precursor molecule as a sulfur provider for the cytosolic CIA Fe-S cluster assembly pathway (29). The ISC pathway thus not only governs the proper assembly of mitochondrial Fe-S proteins, but also of cytoplasmic and nuclear ones. Our quantitative proteomics data suggests it is also the case in *T. gondii*, as several putative nuclear Fe-S proteins involved in gene transcription (such as DNA-dependent RNA polymerases) or DNA repair (like DNA endonulease III) were found to be impacted by TgIscU depletion. The CIA pathway has recently been shown to be important for tachyzoite proliferation (30), and several of the cytoplasmic or nuclear Fe-S cluster-containing proteins are likely essential for parasite viability. It is thus possible that in spite of their conversion to a stress-resistant form, the long-term viability of TgIscU parasites could be affected beyond recovery.

Our quantitative proteomics analysis shows that SUF-impaired parasites also seem to initiate an upregulation of some bradyzoite markers early after TgSufS depletion. Yet, these parasites did not display the hallmarks of bradyzoite morphology. They did not progress towards stage conversion and instead they eventually died. Both the apicoplast and the mitochondrion have established a close metabolic symbiosis with their host cell, so there are likely multiple mechanisms allowing these organelles to communicate their status to the rest of the cell. This raises the question as to why mitochondrion, but not apicoplast, dysfunction can lead to differentiation into bradyzoites. This may be due to differences in the kinetics or the severity of apicoplast-related phenotypes that may not allow stage conversion (which is typically a long process) to happen. Alternatively, there might be differentiation signals specifically associated to the mitochondrion. In fact this organelle is increasingly seen as a signalling platform, able to communicate its fitness through the release of specific metabolites, reactive oxygen species, or by modulating ATP levels (95). Interestingly, it was shown in other eukaryotes that mitochondrial dysfunctions such as altered oxidative phosphorylation significantly impair cellular proliferation, oxygen sensing or specific histone acetylation, yet without diminishing cell viability and instead may lead to quiescent states (96, 97). Environmental and metabolic cues likely drive specific gene expression, leading to a functional shift to drive stage conversion, but how are these stimuli integrated is largely unknown. A high-throughput approach has allowed the recent identification of a master transcriptional regulator of stage conversion (98), but how upstream events are converted into cellular signals to mobilize the master regulator is still an important, yet unresolved, question. Translational control (99) may play a role in regulating this factor in the context of the integrated stress response (100). In fact, an essential part of the eukaryotic cell stress response occurs post-transcriptionally and is achieved by RNA-binding proteins (101). Interestingly, among the proteins significantly less abundant in the mitochondrial SUF pathway mutant were many RNA-binding proteins, including components of stress granules (PolyA-binding protein, PUF1, Alba1 and 2, some of which are linked to stage conversion (102–104)) which are potentially involved in mRNA sequestration from the translational machinery, but also two regulators of the large 60S ribosomal subunit assembly, as well as the gamma subunit of the eukaryotic translation initiation factor (eIF) complex 4 (known to be down-regulated in the bradyzoite stage (105)). Variation in these candidates may have a considerable impact on the translational profile and on the proteostasis of differentiating parasites, and how they may help regulating stage conversion in this context should be investigated further. Understanding the mechanisms that either lead to encystment or death of the parasites is crucial to the development of treatments against toxoplasmosis. This question is key to the pathology caused by *T. gondii*, as bradyzoites act as reservoirs susceptible to reactivate as and cause acute symptoms, and are essentially resistant to treatment. Comparative studies of stress-induced or spontaneously differentiating conditional mutants may bring further insights on how the parasites integrate upstream stresses or dysfunctions into global regulation of stage conversion.

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Materials and methods

Parasites and cells culture. Tachyzoites of the TATi ΔKu80 *T. gondii* strain (46), as well as derived transgenic parasites generated in this study, were maintained by serial passage in human foreskin fibroblast (HFF, American Type Culture Collection, CRL 1634) cell monolayer grown in Dulbecco's modified Eagle medium (Gibco), supplemented with 5% decomplemented fetal bovine serum, 2-mM L-glutamine and a cocktail of penicillin-streptomycin at 100 µg/ml.

Bioinformatic analyses. Sequence alignments were performed using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm of the Geneious 6.1.8 software suite (http://www.geneious.com). Transit peptide and localization predictions were done using IPSORT (http://ipsort.hgc.jp/), Localizer 1.0.4 (http://localizer.csiro.au/), and Deeploc 1.0 (http://www.cbs.dtu.dk/services/DeepLoc-1.0/) algorithms.

The putative Fe-S proteome was predicted using the MetalPredator webserver (http://metalweb.cerm.unifi.it/tools/metalpredator/) (56). The whole complement of *T. gondii* annotated proteins was downloaded in FASTA format from the ToxoDB database (https://toxodb.org (41), release 45) and used for analysis in the MetalPredator webserver. Additional manual curation included homology searches for known Fe-S proteins from plants (see appendix A in (106)), and search for homologues in the Uniprot database (https://www.uniprot.org) that were annotated as containing a Fe-S cofactor. For proteomics candidates, annotations were inferred from ToxoDB, KEGG (https://www.genome.jp/kegg/) and the Liverpool Library of Apicomplexan Metabolic Pathways (http://www.llamp.net/ (107)). N-glycosylation predictions were done with the GlycoEP webserver (http://crdd.osdd.net/raghava/glycoep/index.html). GPI anchor predictions were done with the PredGPI (http://gpcr.biocomp.unibo.it/predgpi/) and GPI-SOM (http://gpi.unibe.ch/) webservers.

Heterologous expression in *E. coli*. Constructs for designing recombinant proteins were defined by aligning TgSufS and TgIscU amino acid sequences with their *E. coli* counterparts. For *TgSufS*, a 1,438 bp fragment corresponding to amino acids 271-699, was amplified by polymerase chain reaction (PCR) from *T. gondii* cDNA using primers ML4201/ML4012 (sequences of the primers used in this study are found in Table S6). For *TgIscU*, a 393 bp fragment corresponding to amino acids 64-194, was amplified by PCR from *T. gondii* cDNA using primers ML4204/ML4205. The fragments were cloned into the pUC19 (Thermo Fisher Scientific) using the HindIII/BamHI and SphI/BamHI restriction sites, respectively. *E. coli* mutants from the Keio collection (obtained from the The *Coli* Genetic Stock Center at the University of Yale: stain numbers JW1670-1 for *SufS*, JW2513-1 for *IscU*), were transformed with plasmids for expressing recombinant TgSufS and TgIscU and selected with ampicillin. For growth assays (108), overnight stationary phase cultures were adjusted to the same starting OD_{600} of 0.6 in salt-supplemented M9 minimal media containing 0.4% glucose and varying amounts of the 2,2-Bipyridyl iron chelator (Sigma-Aldrich). Growth was monitored through OD_{600} measurement after 7, 14 and 24 hours at 37°C in a shaking incubator.

Generation of HA-tagged TgSufS, TgSufE2 and TgIscU cell lines. The ligation independent strategy (45) was used for C-terminal hemagglutinin $(HA)_3$-tagging TgIscU. Fragment corresponding to the 3' end of the target gene was amplified by PCR from genomic DNA, with the Q5 DNA polymerase (New England BioLabs) using primers ML4208/ML4209 (*TgIscU*) and inserted in frame with the sequence coding for a triple HA tag, present in the pLIC-HA_3-chloramphenicol acetyltransferase (CAT) plasmid. The resulting vector was linearized and 40 µg of DNA was transfected into the TATi ΔKu80 cell line to

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allow integration by single homologous recombination, and transgenic parasites of the TgIscU-HA cell line were selected with chloramphenicol and cloned by serial limiting dilution.

For TgSufS and TgSufE2, a CRISPR-based strategy was used. Using the pLIC-HA_3-CAT plasmid as a template, a PCR was performed with the KOD DNA polymerase (Novagen) to amplify the tag and the resistance gene expression cassette with primers ML3978/ML3979 (*TgSufS*) and ML4023/ML4162 (*TgSufE2*), that also carry 30 bp homology with the 3 end of the corresponding genes. A specific single-guide RNA (sgRNA) was generated to introduce a double-stranded break at the 3 of the respective loci. Primers used to generate the guides were ML3948/ML3949 (*TgSufS*) and ML4160/ML4161 (*TgSufE2*) and the protospacer sequences were introduced in the Cas9-expressing pU6-Universal plasmid (Addgene, ref #52694) (43). Again, the TATi ΔKu80 cell line was transfected and transgenic parasites of the TgSufS-HA or TgSufE2-HA cell lines were selected with chloramphenicol and cloned by serial limiting dilution.

Generation of TgSufS and TgIscU conditional knock-down and complemented cell lines. The conditional knock-down cell for *TgSufS* and *TgIscU* were generated based on the Tet-Off system using the DHFR-TetO7Sag4 plasmid (109).

For *TgIscU*, a 930 bp 5' region of the gene, starting with the initiation codon, was amplified from genomic DNA by PCR using Q5 polymerase (New England Biolabs) with primers ML4212/ML4213 and cloned into the DHFR-TetO7Sag4 plasmid, downstream of the anhydrotetracycline (ATc)-inducible TetO7Sag4 promoter, obtaining the DHFR-TetO7Sag4-TgIscU plasmid. The plasmid was then linearized and transfected into the TgIscU-HA cell line. Transfected parasites were selected with pyrimethamine and cloned by serial limiting dilution.

For *TgSufS*, a CRISPR-based strategy was used. Using the DHFR-TetO7Sag4 plasmid as a template, a PCR was performed with the KOD DNA polymerase (Novagen) to amplify the promoter and the resistance gene expression cassette with primers ML4154/ML4155 that also carry 30 bp homology with the 5 end of the *TgSufS* gene. A specific single-guide RNA (sgRNA) was generated to introduce a double-stranded break at the 5 of the *TgSufS* locus. Primers used to generate the guide were ML4156/ML4157 and the protospacer sequences were introduced in the pU6-Universal plasmid (Addgene ref#52694) (43). The TgSufS-HA cell line was transfected with the donor sequence and the Cas9/guide RNA-expressing plasmid and transgenic parasites were selected with pyrimethamine and cloned by serial limiting dilution.

The cKD TgSufS-HA and cKD TgIscU-HA cell lines were complemented by the addition of an extra copy of the respective genes put under the dependence of a tubulin promoter at the *uracil phosphoribosyltransferase* (*UPRT*) locus. *TgSufS* (2097 bp) and *TgIscU* (657 bp) whole cDNA sequences were amplified by reverse transcription (RT)-PCR with primers ML4576/ML4577 and ML4455/ML4456, respectively. They were then cloned downstream of the *tubulin* promoter sequence of the pUPRT-TUB-Ty vector (46) to yield the pUPRT-TgSufS and pUPRT-TgIscU plasmids, respectively. These plasmids were then linearized prior to transfection of the respective mutant cell lines. The recombination efficiency was increased by co-transfecting with the Cas9-expressing pU6-UPRT plasmids generated by integrating *UPRT*-specific protospacer sequences (with primers ML2087/ML2088 for the 3' and primers ML3445/ML3446 for the 5') which were designed to allow a double-strand break at the *UPRT* locus. Transgenic parasites were selected using 5-fluorodeoxyuridine and cloned by serial limiting dilution to yield the cKD TgSufS-HA comp cKD TgIscU-HA comp cell lines, respectively.

Immunoblot analysis. Protein extracts from 10^7 freshly egressed tachyzoites were prepared in Laemmli sample buffer, separated by SDS-PAGE and transferred onto nitrocellulose membrane using

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the BioRad Mini-Transblot system according to the manufacturer's instructions. Rat monoclonal antibody (clone 3F10, Roche) was used to detect HA-tagged proteins. Other primary antibodies used were rabbit anti-lipoic acid antibody (ab58724, Abcam), mouse anti-SAG1 (110), rabbit anti-CPN60 (111) and mouse anti-actin (112).

Immunofluorescence microscopy. For immunofluorescence assays (IFA), intracellular tachyzoites grown on coverslips containing HFF monolayers, were either fixed for 20 min with 4% (w/v) paraformaldehyde in PBS and permeabilized for 10 min with 0.3% Triton X-100 in PBS or fixed for 5 min in cold methanol (for the use of cyst-specific antibodies). Slides/coverslips were subsequently blocked with 0.1% (w/v) BSA in PBS. Primary antibodies used (at 1/1,000, unless specified) to detect subcellular structures were rabbit anti-CPN60 (111), mouse monoclonal anti-F1-ATPase beta subunit (gift of P. Bradley), mouse monoclonal anti-GRA3 (113), rabbit anti-GAP45 (114), mouse monoclonal anti-SAG1 (110), anti SAG4/P18 (diluted 1/200, T8 3B1) and anti P21 (diluted 1/200, T8 4G10) (115). Rat monoclonal anti-HA antibody (clone 3F10, Roche) was used to detect epitope-tagged proteins. Staining of DNA was performed on fixed cells by incubating them for 5 min in a 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) solution. All images were acquired at the Montpellier RIO imaging facility from a Zeiss AXIO Imager Z1 epifluorescence microscope driven by the ZEN software v2.3 (Zeiss). Z-stack acquisition and maximal intensity projection was performed to visualize larger structures such as in vitro cysts. Adjustments for brightness and contrast were applied uniformly on the entire image.

Plaque assay. Confluent monolayers of HFFs were infected with freshly egressed parasites, which were left to grow for 7 days in the absence or presence of ATc. They were then fixed with 4% v/v paraformaldehyde (PFA) and plaques were revealed by staining with a 0.1% crystal violet solution (V5265, Sigma-Aldrich).

Egress assay. *T. gondii* tachyzoites were grown for 40 (without ATc) or 120 (with ATc) hours on HFF cells with coverslips in 24-well plates. The infected host cells were incubated for 7 min at 37°C with DMEM containing 3 µM of calcium ionophore A23187 (C7522, Sigma-Aldrich) prior to fixation with 4% PFA. Immunofluorescence assays were performed as previously described (116): the parasites and the parasitophorous vacuole membrane were labelled with anti-GAP45 and anti-GRA3, respectively. The proportion of egressed and non-egressed vacuoles was calculated by counting 250 vacuoles in three independent experiments. Data are presented as mean values ± SEM.

Semi-quantitative RT-PCR. Total mRNAs of freshly egressed extracellular parasites from the cKD TgSufS-HA, cKD TgIscU-HA and their respective complemented cell lines (incubated with or without ATc at 1.5 µg/mL for 3 days) were extracted using Nucleospin RNA II Kit (Macherey-Nagel). The cDNAs were synthesized with 450 ng of total RNA per RT-PCR reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Specific primers for *TgSufS* (ML4686/ML4687), *TgIscU* (ML4684/ML4685) and, as a control, *Tubulin β* (ML841/ML842) were used to amplify specific transcripts with the GoTaq DNA polymerase (Promega). PCR was performed with 21 cycles of denaturation (30 s, 95 °C), annealing (20 s, 55 °C), and elongation (30 s, 72 °C).

Mitochondrial membrane potential measurement. Parasites grown for the indicated time with or without ATc were mechanically released from their host cells, purified on a glass wool fiber column, washed and adjusted to 10^7 parasites/ml in phenol red-free medium, and incubated in with 1.5 µM of the JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine Iodide, T3168, Invitrogen) for 30 min at 37°C, washed phenol red-free medium and analyzed by flow cytometry or microscopy. Flow cytometry analysis was performed on a FACSAria III flow cytometer (Becton

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Dickinson). An unstained control was used to define gates for analysis. 50,000 events per condition were collected and data were analysed using the FlowJo Software.

Quantitative label-free mass spectrometry. Parasites of the TATi ΔKu80 and cKD TgIscU-HA cell lines were grown for two days in the presence of ATc; parasites of the cKD TgSufS-HA were grown for three days in the presence of ATc. Then they were mechanically released from their host cells, purified on a glass wool fiber column, washed in Hanks' Balanced Salt Solution (Gibco). Samples were first normalized on parasite counts, but further adjustment was performed after parasite pellet resuspension in SDS lysis buffer (50 mm Tris-HCl pH8, 10 mm EDTA pH8, 1% SDS) and protein quantification with a bicinchoninic acid assay kit (Abcam). For each condition, 20 µg of total proteins were separated on a 12% SDS-PAGE run for 20 min at 100 V, stained with colloidal blue (Thermo Fisher Scientific), and each lane was cut in three identical fractions. Trypsin digestion and mass spectrometry analysis in the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) were carried out as described previously (117).

For peptide identification and quantification, the raw files were analyzed with MaxQuant version 1.6.10.43 using default settings. The minimal peptide length was set to 6. Carbamidomethylation of cysteine was selected as a fixed modification and oxidation of methionine, N-terminal-pyroglutamylation of glutamine and glutamate and acetylation (protein N terminus) as variable modifications. Up to two missed cleavages were allowed. The files were searched against the *T. gondii* proteome (March 2020 -https: //www.uniprot.org/proteomes/UP000005641-8450 entries). Identified proteins were filtered according to the following criteria: at least two different trypsin peptides with at least one unique peptide, an *E* value below 0.01 and a protein E value smaller than 0.01 were required. Using the above criteria, the rate of false peptide sequence assignment and false protein identification were lower than 1%. Peptide ion intensity values derived from MaxQuant were subjected for label-free quantitation. Unique and razor peptides were considered (118). Statistical analyses were carried out using R package software. ANOVA test with threshold of 0.05 was applied to identify the significant differences in the protein abundance. Hits were retained if they were quantified in at least three of the four replicates in at least one experiment. Additional candidates that consistently showed absence or presence of LFQ values versus the control, and mean LFQ was only considered if peptides were detected in at least 3 out of the four biological replicates.

Statistical analysis for phenotypic assays. Unless specified, values are usually expressed as means ± standard error of the mean (SEM). Data were analysed for comparison using unpaired Student's t-test with equal variance (homoscedastic) for different samples or paired Student's t-test for similar samples before and after treatment.

Data availability. All raw MS data and MaxQuant files generated have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (https://www.ebi.ac.uk/pride/archive) with the dataset identifier PXD023854.

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1032 **Figure legends**
1033
1034 **Figure 1. TgSufS and TgIscU are functional homologs of components of the plastidic and**
1035 **mitochondrial iron sulfur cluster synthesis pathways.**

bioRxiv preprint doi: https://doi.org/10.1101/2021.01.28.428257; this version posted January 28, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

A) Putative Fe-S cluster synthesis pathways and associated molecular machinery in *Toxoplasma*. B) Functional complementation of bacterial mutants for IscU (top) and SufS (bottom). Growth of bacterial mutant strains and strains complemented ('comp') by their respective *T. gondii* homologues ('comp'), was assessed by monitoring the optical density at 600 nm in the presence or not of an iron chelator (2,2'-bipyridyl, 'chel'). Values are mean from *n*=3 independent experiments ±SEM. * denotes $p \leq 0.05$, Student's *t*-test.

Figure 2. TgSufS and TgIscU localize to the apicoplast and the mitochondrion, respectively. Detection by immunoblot of C-terminally HA-tagged TgSufS (A) and TgIscU (B) in parasite extracts reveals the presence of both precusor (p) and mature (m) forms of the proteins. Anti-actin (TgACT1) antibody was used as a loading control. Immunofluorescence assay shows TgSufS co-localizes with apicoplast marker TgCPN60 (C) and TgIscU co-localizes with mitochondrial marker F1 β ATPase (D). Scale bar represents 5 µm. DNA was labelled with DAPI. DIC: differential interference contrast.

Figure 3. Efficient down-regulation of TgSufS and TgIscU expression with anhydrotetracyclin (ATc). A) Immunoblot analysis with anti-HA antibody shows efficient down-regulation of TgSufS after 48h of incubation with ATc. Anti-SAG1 antibody was used as a loading control. B) Immunoblot analysis with anti-HA antibody shows efficient down-regulation of TgIscU after 24h of incubation with ATc. Anti-SAG1 antibody was used as a loading control. C) and D) Immunofluorescence assays show TgSufS and TgIscU are not detectable anymore after 48h of incubation with ATc. Scale bar represents 5 µm. DNA was labelled with DAPI. DIC: differential interference contrast.

Figure 4. Depletion of TgSufS and TgIscU affects in vitro growth of the tachyzoites. Plaque assays were carried out by infecting HFF monolayers with the TATi ΔKu80 cell line, the cKD TgSufS-HA (A) or the cKD TgIscU-HA (B) cell lines, or parasites complemented with a wild-type version of the respective proteins. They were grown for 7 days ± ATc. Measurements of lysis plaque areas are shown on the right and highlight a significant defect in the lytic cycle when TgSufS (A) or TgIscU (B) were depleted. Values are means of *n*=3 experiments ± SEM. ** denotes $p \leq 0.01$, Student's *t*-test. Scale bars= 1mm. TgSufS (C) and TgIscU (D) mutant and complemented cell lines, as well as their parental cell lines and the TATi ΔKu80 control, were grown in HFF in the presence or absence of ATc for 48 hours, and subsequently allowed to invade and grow in new HFF cells for an extra 24 hours in the presence of ATc. Parasites per vacuole were then counted. Values are means ± SEM from *n*=3 independent experiments for which 200 vacuoles were counted for each condition. E) Plaque assays for the TgSufS and TgIscU mutants were performed as described in A) and B), but ATc was washed out after 7 days and parasites were left to grow for an extra 4 days. Plaque number and area were measured. Data are means ± SEM from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, Student's *t*-test. Arrowheads show plaques forming in the TgIscU upon ATc removal. Scale bar= 1mm.

Figure 5. Impact of TgSufS and TgIscU depletion on intracellular tachyzoites. A) Depletion of TgSufS impacts the apicoplast. cKD TgSufS-HA parasites were kept in the presence of ATc and the aspect of the apicoplast and mitochondrion was evaluated by microscopic observation using specific markers (CPN60 and F1β ATPase, respectively). After 72 hours, parasites egressed and were used to reinvade new host cells for subsequent timepoints. Scale bar represents 5 µm. DNA was labelled with DAPI. DIC: differential interference contrast. B) Quantification of apicoplast loss in vacuoles containing cKD TgSufS-HA parasites after 72 to 120 hours of incubation with ATc. Data are mean values from *n*=3 independent experiments ±SEM. ** $p \leq 0.005$, **** $p \leq 0.0001$, Student's *t*-test. C) Depletion of TgIscU does not impact mitochondrial and overall parasite morphologies, but affects parasite growth. cKD TgIscU-HA parasites were grown in the presence of ATc for up to five days and the aspect of the apicoplast and mitochondrion was evaluated by microscopic observation

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using specific markers described in A). Growth in the presence of ATc was continuous for up to five days. Scale bar represents 5 µm. DNA was labelled with DAPI. DIC: differential interference contrast. D) Egress is not affected by TgIscU depletion. An egress assay was performed using calcium ionophore A23187. On the left are representative images of vacuoles containing parasites that egressed normally or did not. GRA3 (parasitophorous vacuole marker) staining is shown in green and GAP45 (parasite periphery marker) in red. Scale bars= 10µm. On the right is the quantification of egress for cKD TgIscU-HA parasites kept in the presence of ATc or not. Mean values ± SEM from *n*=3 independent biological experiments are represented.

Figure 6. Change in protein expression induced by TgSufS and TgIscU depletion. A) Volcano plots showing the protein expression difference based on label-free quantitative proteomic data from TgSufS ans TgIscU mutants grown in the presence of ATc. X-axis shows log2 fold change versus the TATi ΔKu80 control grown in the same conditions, and the Y-axis shows -log10(p value) after ANOVA statistical test for *n*=4 independent biological replicates. Less abundant or more abundant proteins that were selected for analysis are displayed in red and blue, respectively. B) Venn diagram representation of the shared and unique proteins whose expression is affected by the depletion of TgSufS and TgIscU.

Figure 7. Depletion of TgSufS impacts know apicoplast Fe-S protein function, but also seem to trigger compensatory response from other cellular pathways. Classification of variant proteins according to their putative cellular localization (A) and function (B). N/A: not available; ER: endoplasmic reticulum; PM: plasma membrane; VAC: vacuolar compartment; GRA: dense granule protein; SRS: SAG-related sequence. In particular, the increased expression of ER-located lipid metabolism enzymes suggests possible compensation for loss of apicoplast-related lipid synthesis function. C) A decrease in the lipoylation of the E2 subunit of proline dehydrogenase (TgPDH-E2), which depends on the Fe-S protein LipA lipoyl synthase in the apicoplast, was observed by immunoblot using an anti-lipoic acid antibody on cell extracts from cKD TgSufS-HA parasites kept with ATc for an increasing period of time. TgCPN60 was used as a control for apicoplast integrity. TgSAG1 was used as a loading control.

Figure 8. TgIscU-depleted parasites show a marked decrease in proteins related to mitochondrial respiration, and a strong increase in bradyzoite-specific dense granules proteins and surface antigens. Classification of variant proteins according to their putative cellular localization (A) and function (B). N/A: not available; ER: endoplasmic reticulum; PM: plasma membrane; VAC: vacuolar compartment; GRA: dense granule protein; SRS: SAG-related sequence. A large proportion of components of complexes II, III and IV of the mitochondrial respiratory chain, which involve Fe-S proteins, were found to be less abundant. Conversely, the abundance of many bradyzoite-specific dense granule proteins of plasma membrane-located surface antigens increased.

Figure 9. Depletion of TgIscU stongly impacts the parasite mitochondrial membrane potential. A) TATi ΔKu80 or cKD TgIscU-HA parasites were grown in the presence of ATc, mechanically released from their host cells and labelled with the JC-1 dye. This dye exhibits potential-dependent accumulation in the mitochondrion, indicated by a switch from green fluorescence for the monomeric form of the probe, to a concentration-dependent formation of red aggregates (top left, DNA is labelled with DAPI and shown in blue, scale=1µm). B) TATi ΔKu80 (top series) or cKD TgIscU-HA parasites (bottom series) were then analysed by flow cytometry. Unlabelled parasites (no JC-1) was used as a control for gating. One representative experiment out of *n*=3 biological replicates is shown.

Figure 10. Depletion of TgIscU triggers parasite differentiation.

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A) cKD TgIscU-HA parasites were grown in the presence of ATc and labelled with ant-TgIMC3 (to outline parasites and spot dividing parasites) and a lectin of *Dolicos biflorus* (DBL) to specifically outline cyst walls. Scale bar represents 10 µm. DNA was labelled with DAPI. DIC: differential interference contrast. B) Quantification of DBL-positive vacuoles after 24 hours or 48 hours of culture of 1) the cKD TgIscU-HA mutant in the presence of ATc 2) the TATi ΔKu80 cell line, as a negative control, 3) the TATi ΔKu80 cell line in the presence of 100µM nitric oxide (NO), as a positive control. Data are from n=3 independent experiments. Values are mean ±SEM. * denotes $p \leq 0.05$, Student's t-test C) Clustering of bradyzoite (Bz) or tachyzoite (Tz)-specific proteins of the SRS family shows specific enrichment of bradyzoite proteins upon TgIscU depletion. D) The cKD TgIscU-HA mutant was grown for up to 20 days in the presence of ATc and labelled for tachyzoite marker SAG1), or early (P18/SAG4) or late (P21) bradyzoite markers. Scale bar represents 10 µm. DNA was labelled with DAPI. DIC: differential interference contrast. E) Measurement of the cyst area size after growing the cKD TgIscU-HA mutant for 7 and 20 days in the presence of ATc and labelling the cyst wall with DBL and measuring the surface of 60 cysts per condition. Mean ±SD is represented. One representative experiment out of n=3 independent biological replicates is shown. **** denotes $p \leq 0.0001$, Student's t-test.

Supplemental table legends

Table S1. Predicted Toxoplasma homologues of the iron sulfur cluster synthesis machinery. Homology searches were conducted in ToxoBD.org using *Arabidopsis thaliana* proteins as a query. Putative subcellular localization was obtained from the hyperLOPIT data available on ToxoDB.org, or by manual annotation. CRISPR fitness score data was obtained from ToxoDB.org.

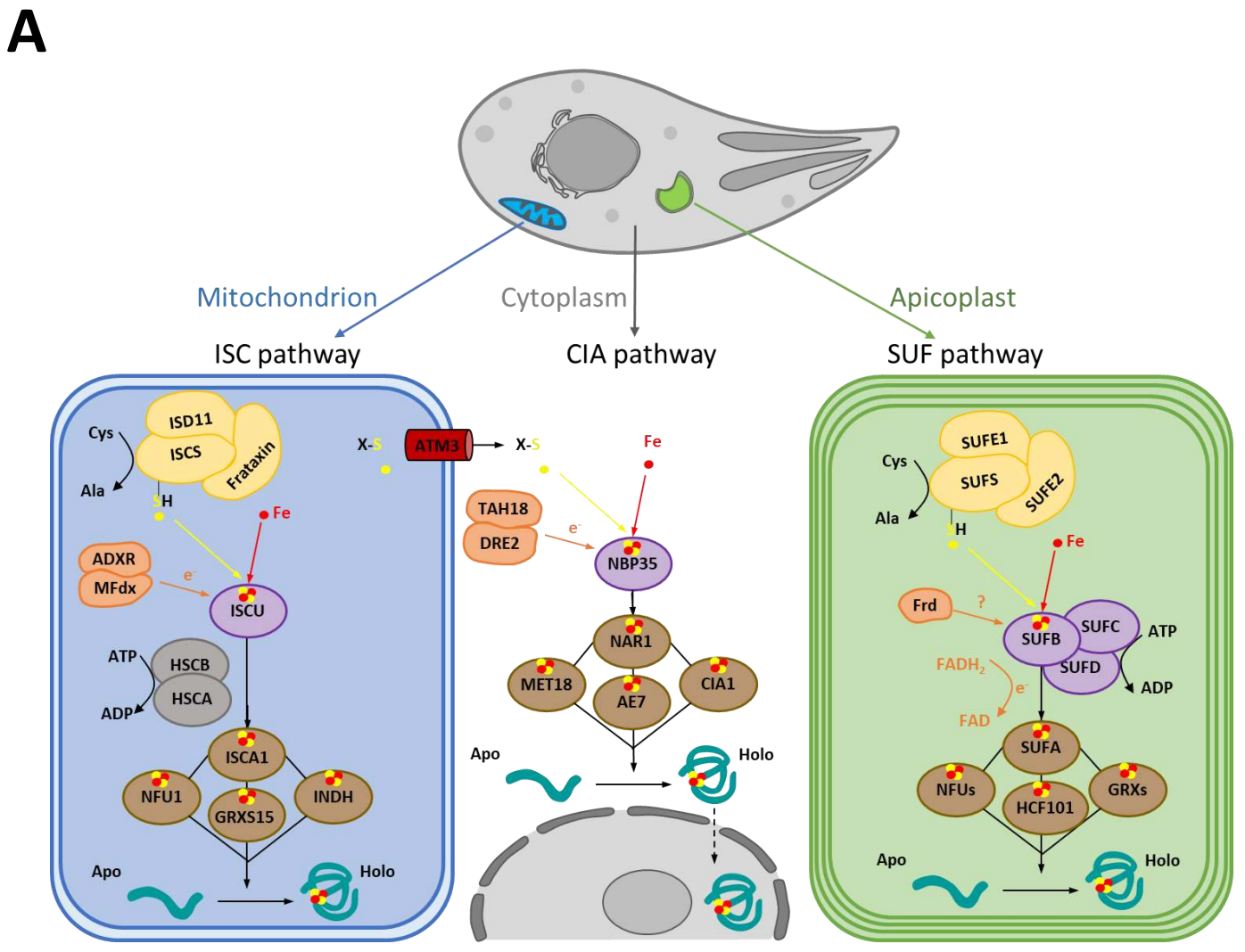
Table S2. Predicted Toxoplasma iron sulfur proteome. The Toxoplasma predicted whole proteome was obtained from the ToxoDB.org database and searched for putative iron sulfur-containing proteins with the MetalPredator web server (http://metalweb.cerm.unifi.it/tools/metalpredator/). Putative subcellular localization was obtained from the hyperLOPIT data available on ToxoDB.org, or by manual annotation. CRISPR fitness score data was obtained from ToxoDB.org.

Table S3. Proteins with lower or higher expression upon depletion of TgSufS as found by label-free quantitative proteomics. For each protein candidate (with www.ToxoDB.org and www.Uniprot.org identifier), $\log_2$ of the different ratio were calculated between the mean MaxQuant LFQ values ('moyLFQ') found for the IscU ('Mito') and SufS ('Apicoplast') mutants, and the TATi ΔKu80 control ('CTRL'). -$\log_{10}$(pvalue) is also provided. Putative subcellular localization was obtained from the hyperLOPIT data available on ToxoDB.org, or by manual annotation. CRISPR fitness score and transcriptomic data for tachyzoites (Tz) and bradyzoites (Bz) were obtained from ToxoDB.org.

Table S4. Proteins with lower or higher expression upon depletion of TgIscU as found by label-free quantitative proteomics. See legend of Table S3. Candidates from the Fe-S proteome (Table S2) that were found to have a lower expression upon TgIscU depletion are highlighted in red.

Table S5. Common proteins with lower or higher expression upon depletion of TgSufS or TgIscU, as found by label-free quantitative proteomics. See legend of Table S3.

Table S6. Oligonucleotides used in this study.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e⁻
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INDH
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MET18
AE7
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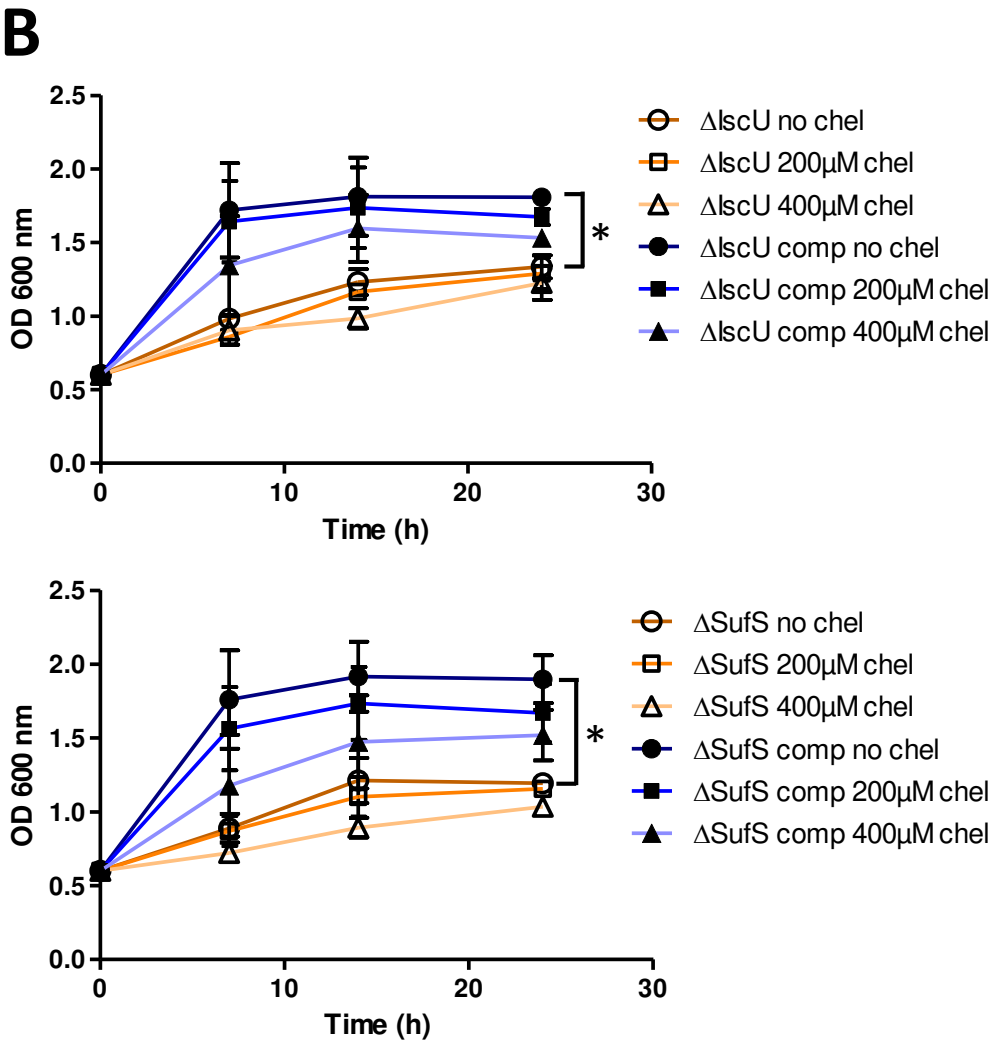
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ΔIscU comp 400μM chel
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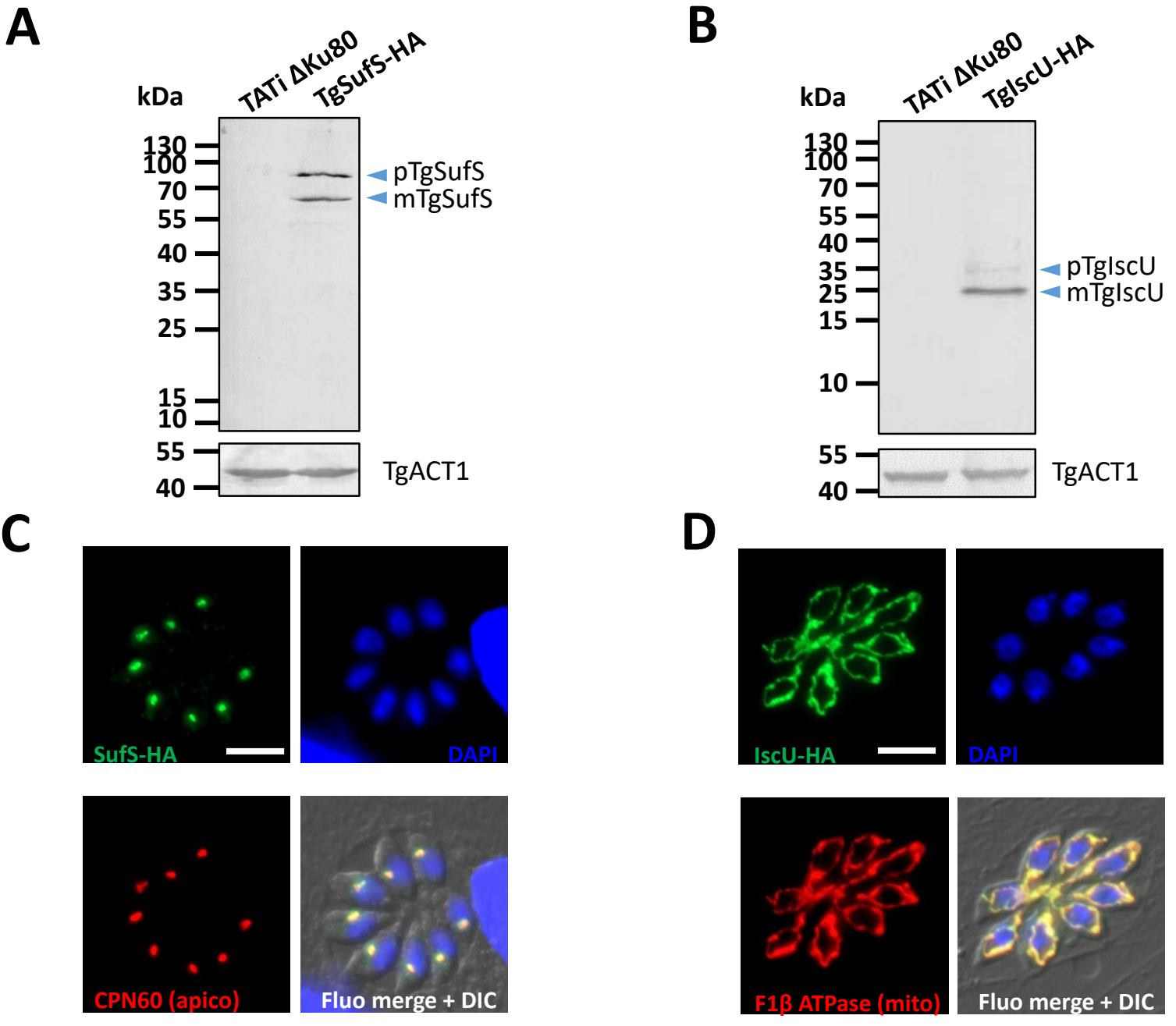
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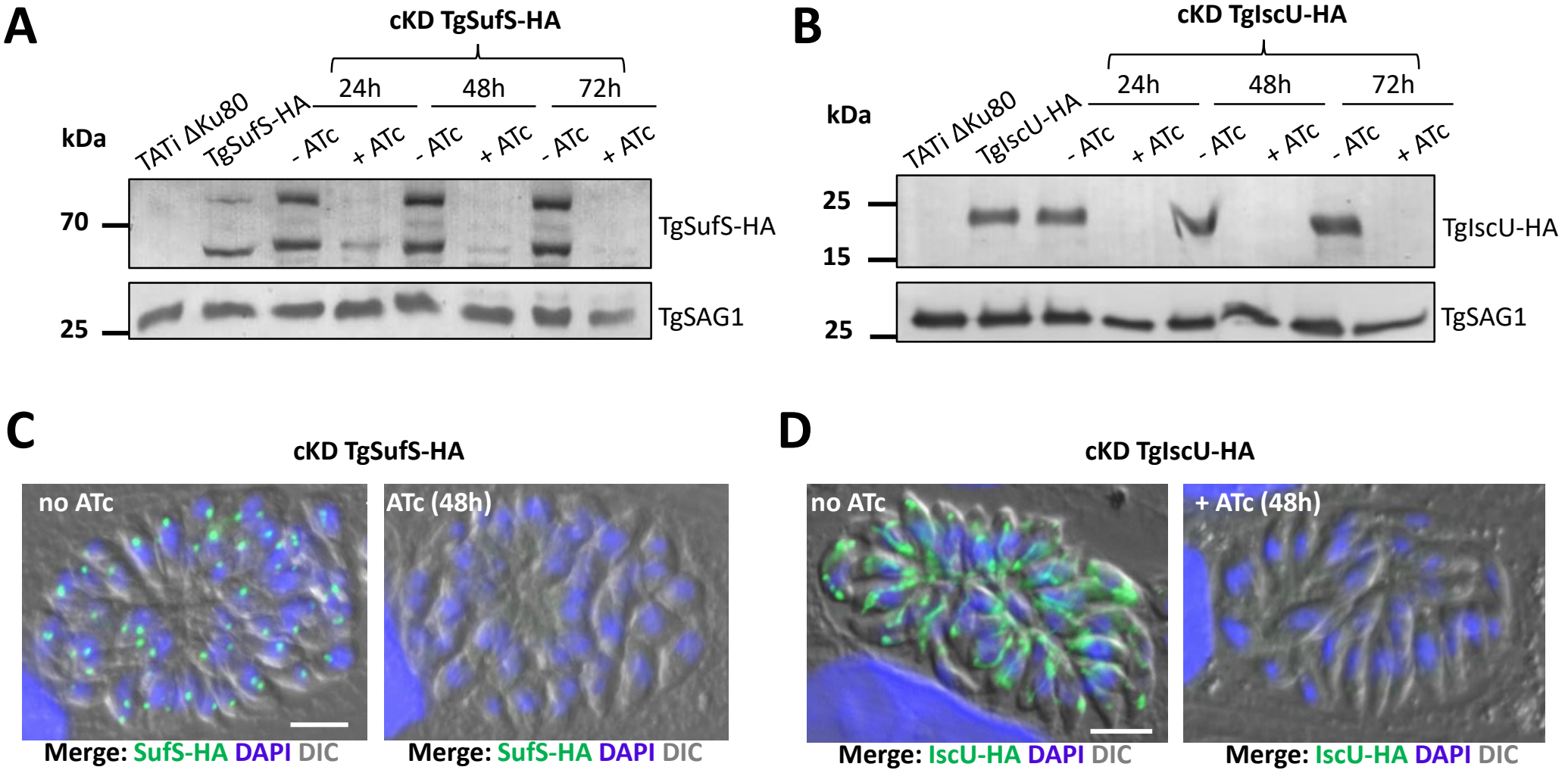
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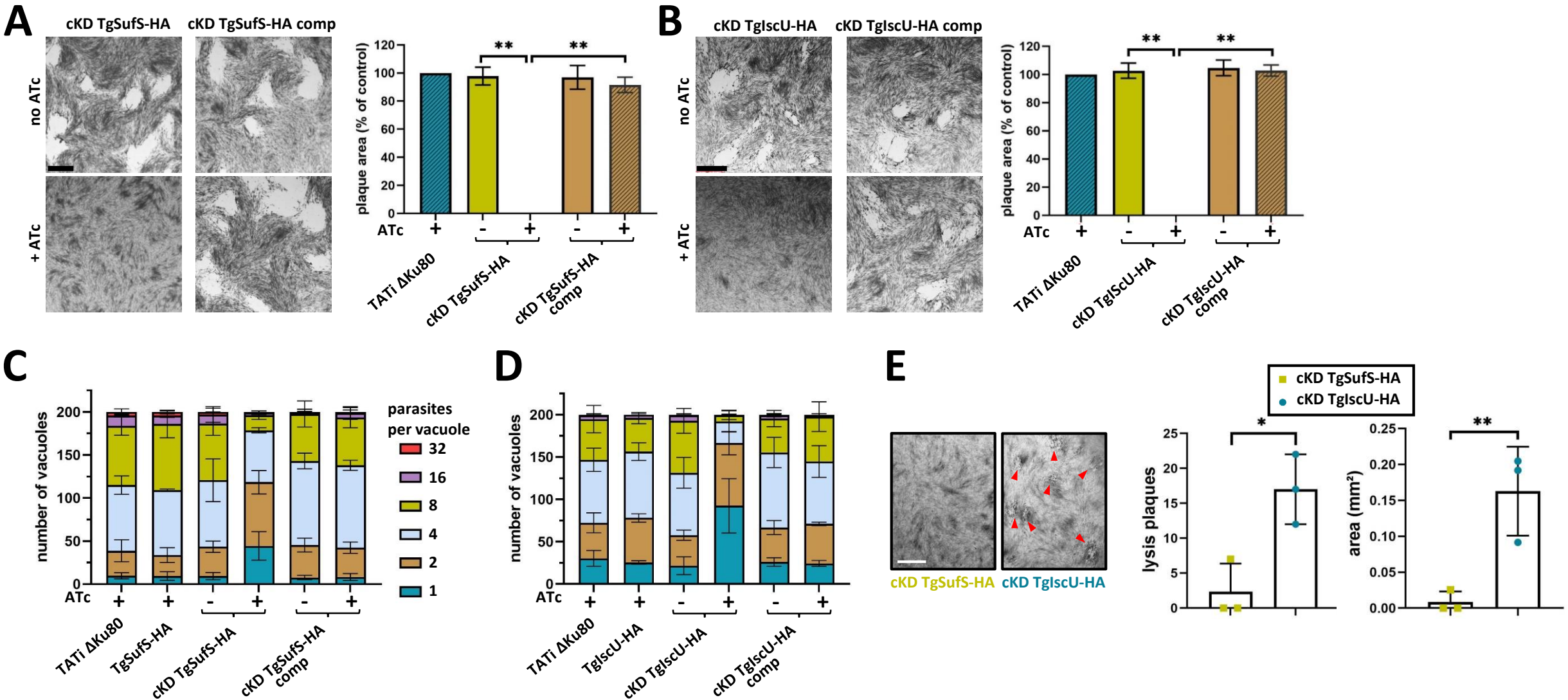
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number of vacuoles
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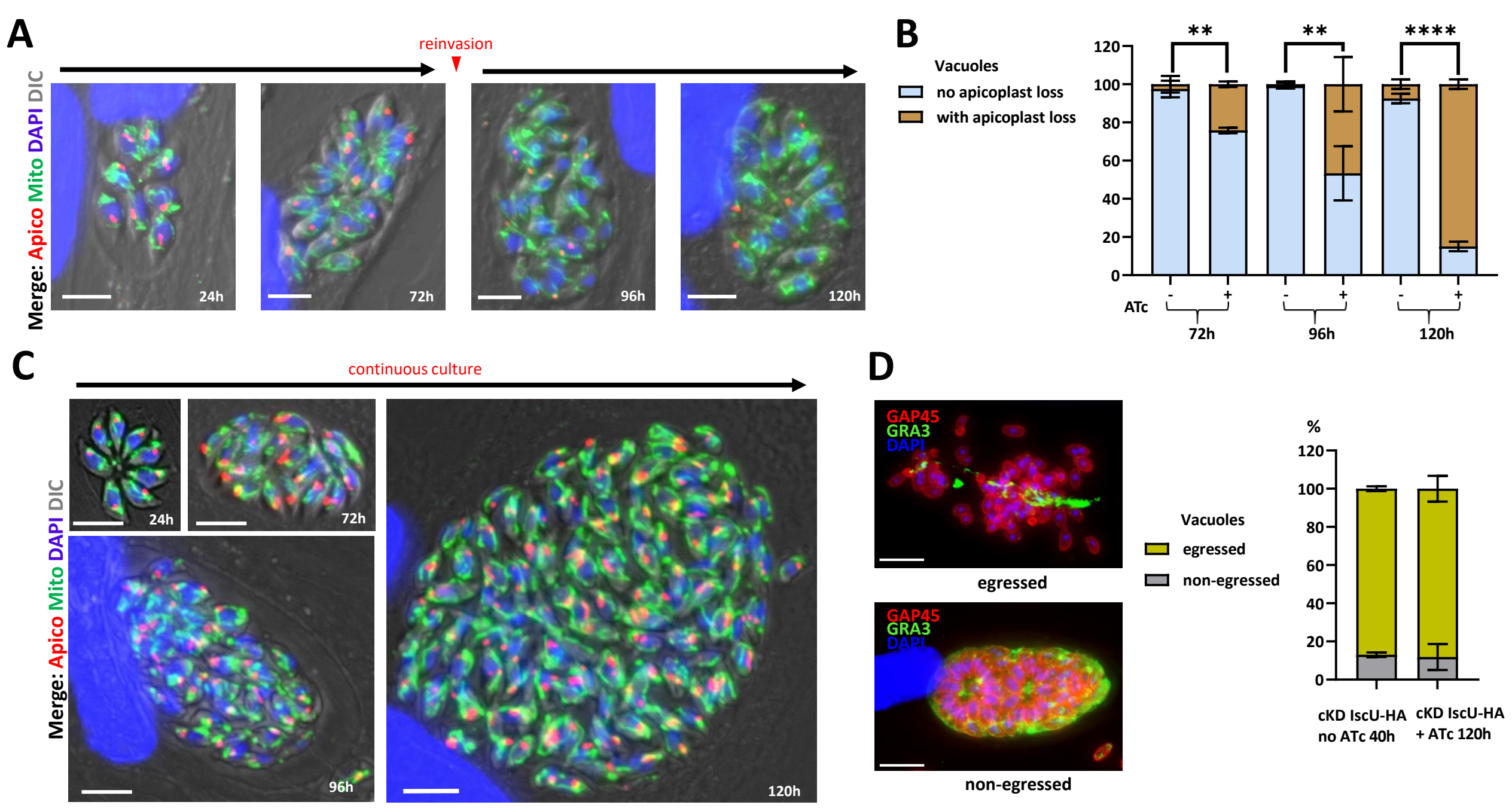
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continuous culture
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GAP45
GRA3
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egressed
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Vacuoles
egressed
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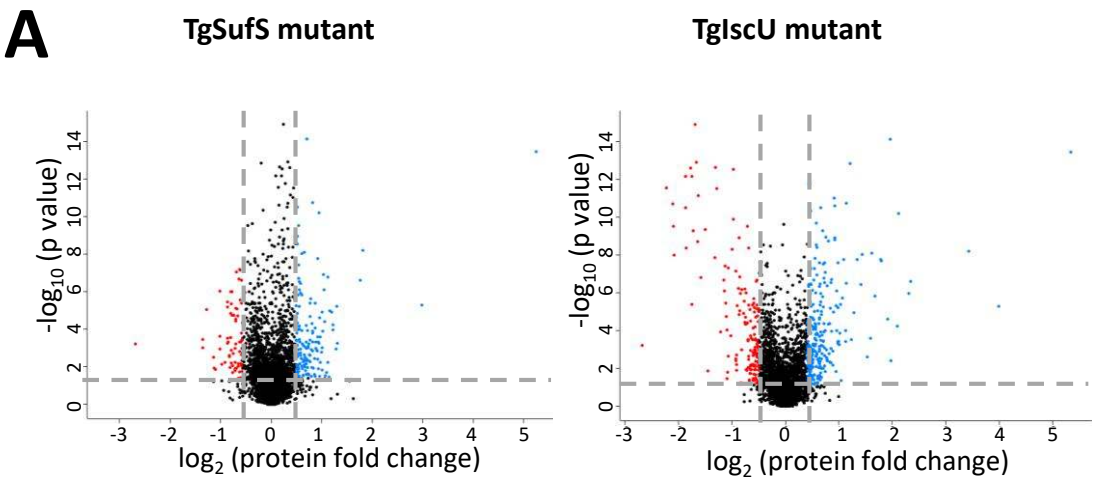
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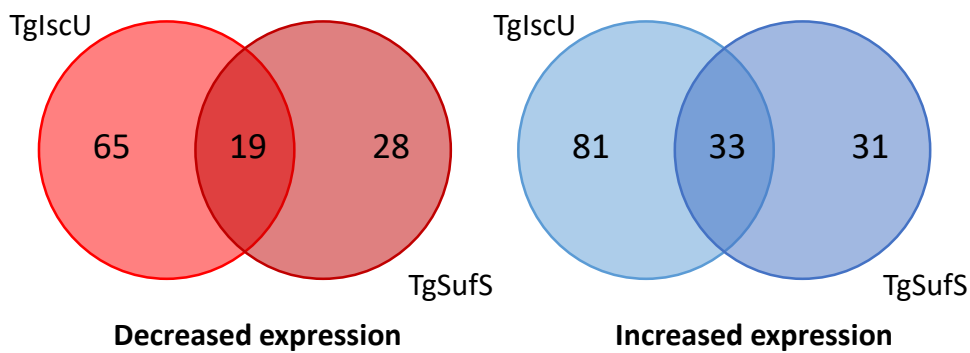
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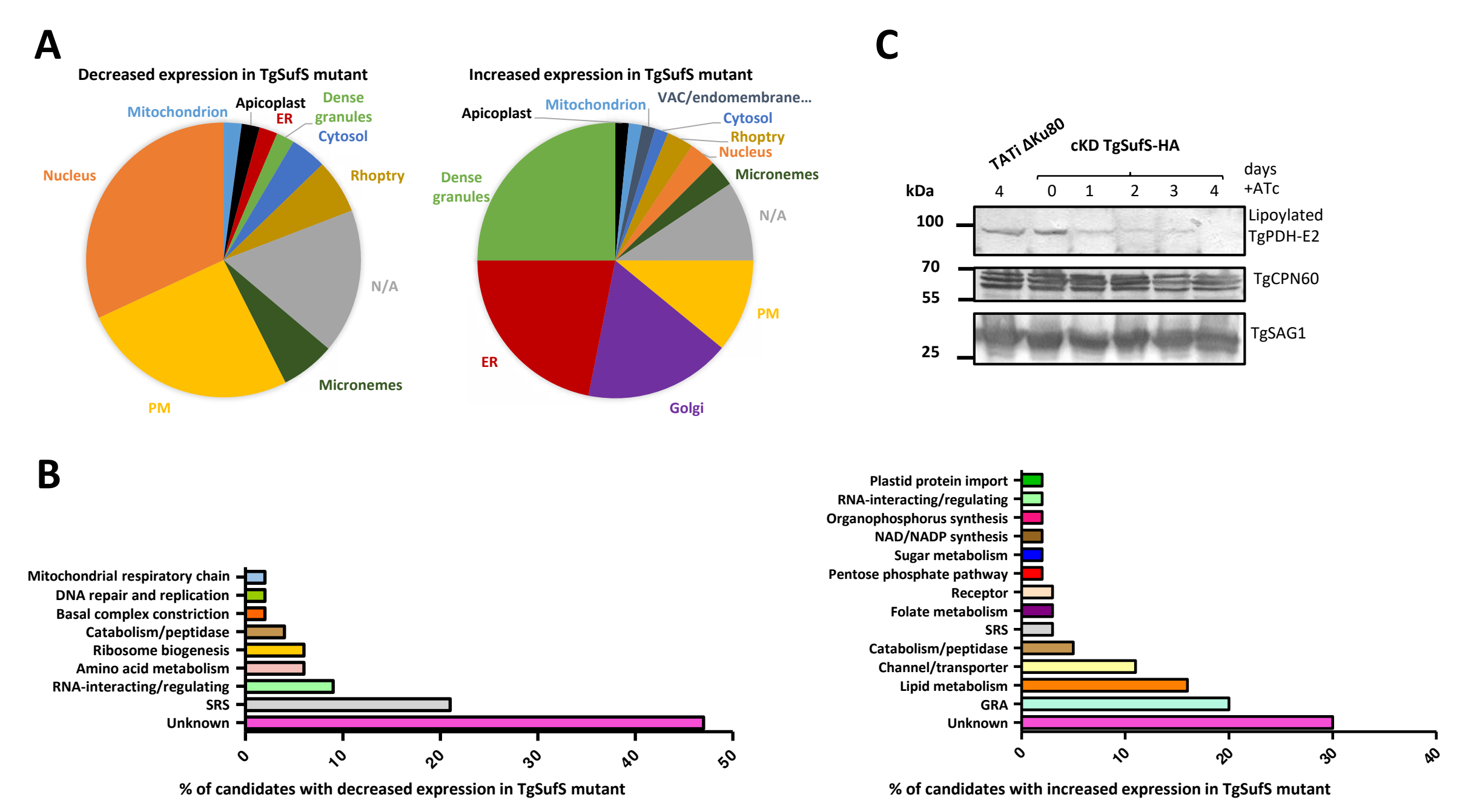
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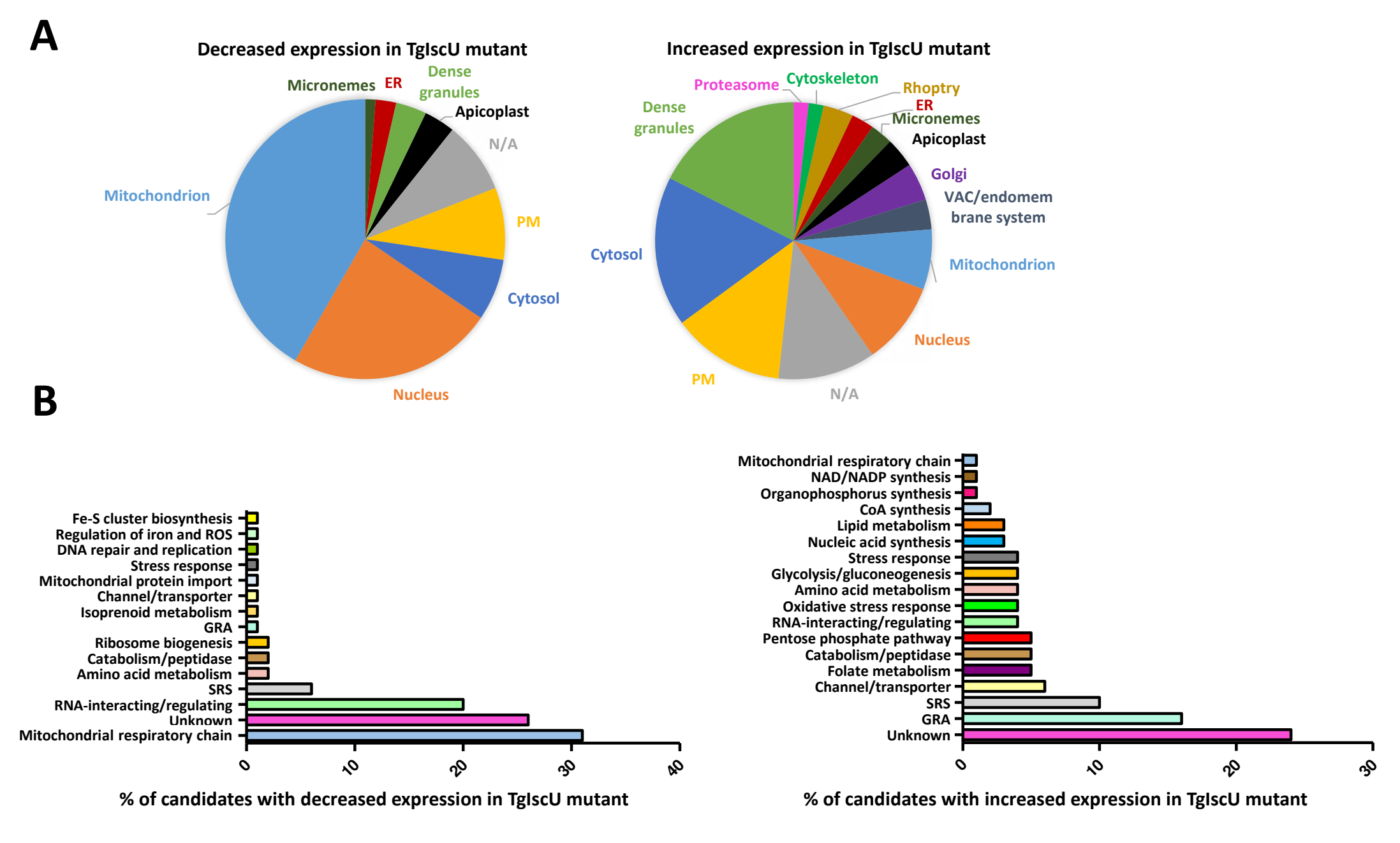
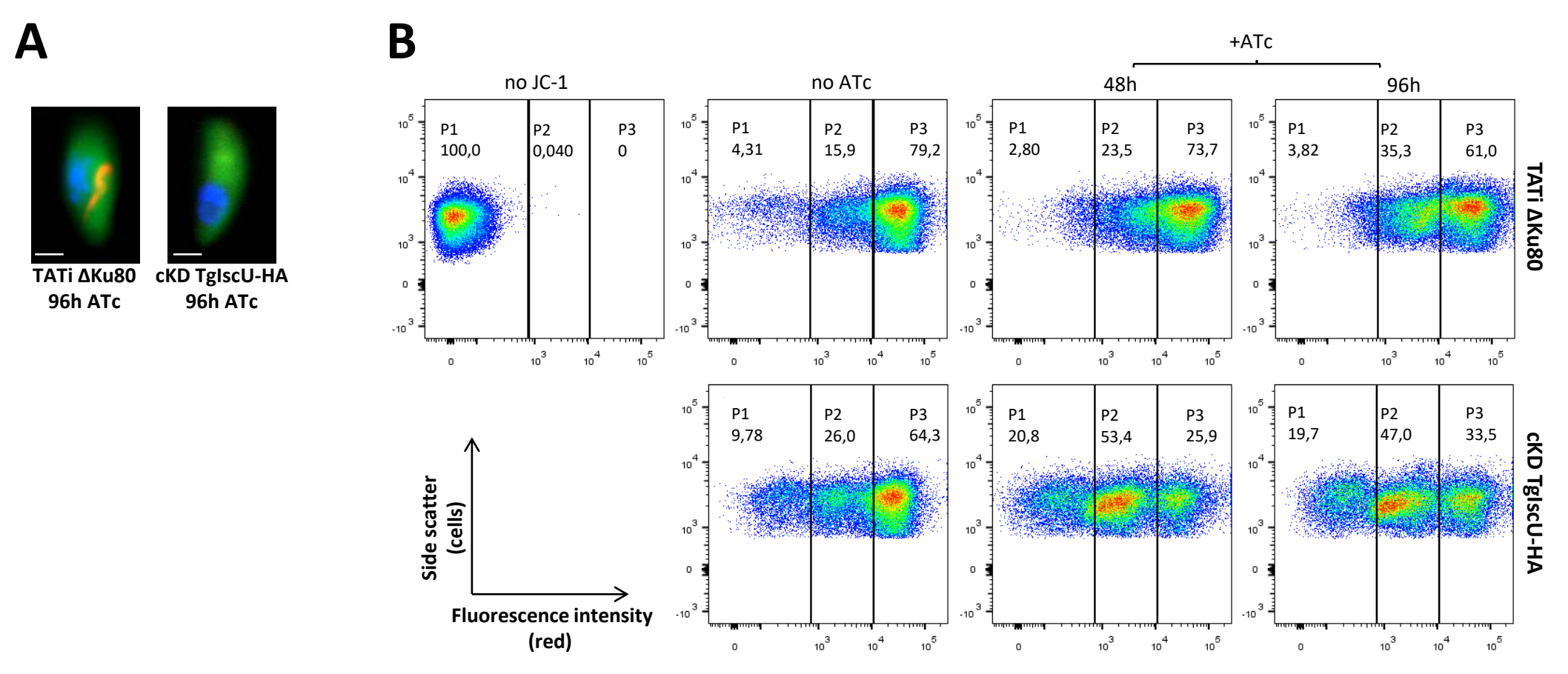
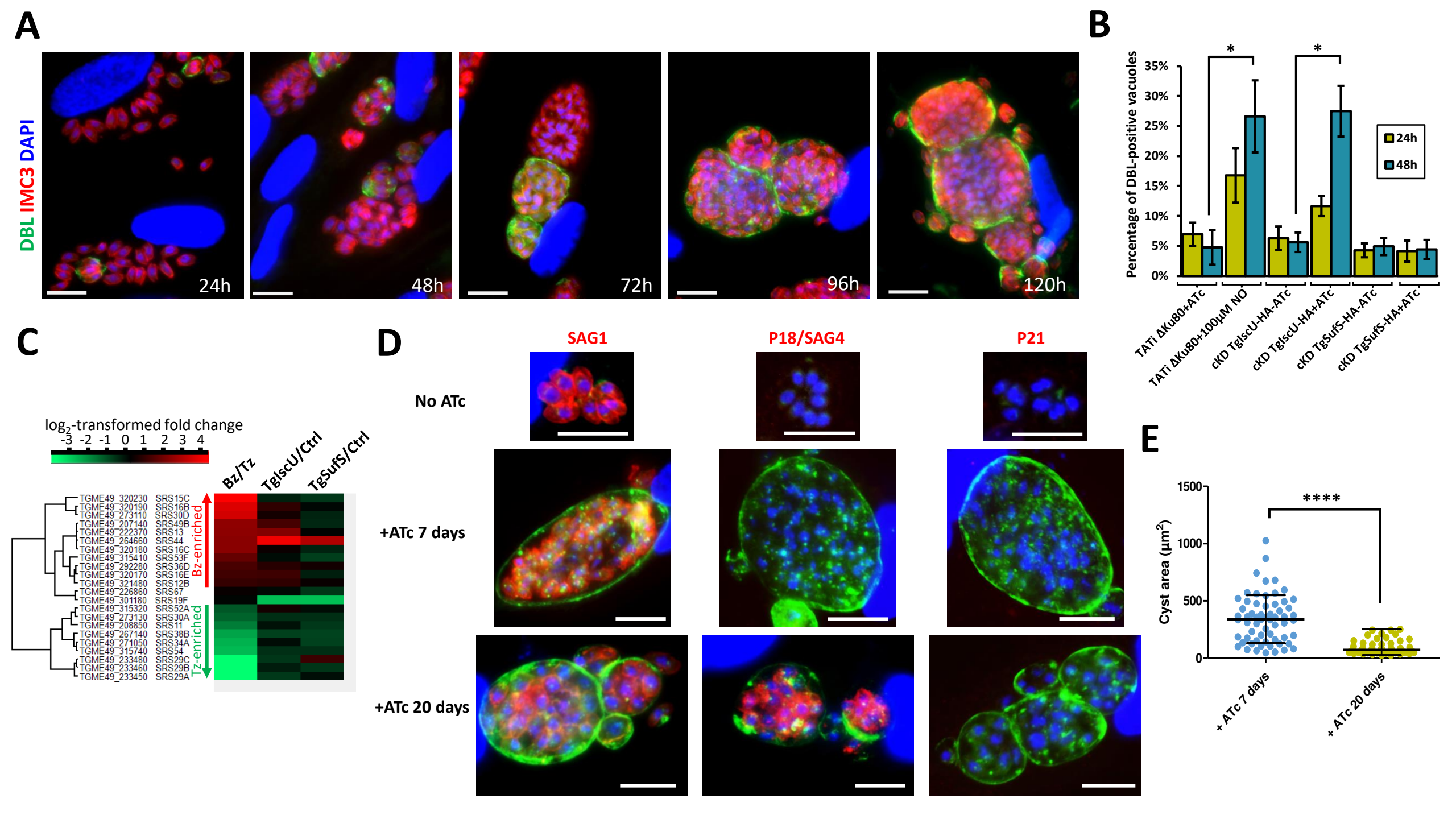
Nucleus
Increased expression in TgSufS mutant
Apicoplast
Mitochondrion
VAC/endomembrane...
Cytosol
Rhoptry
Nucleus
Micronemes
N/A
PM
Golgi
ER
Dense granules
C
TATi ΔKu80
cKD TgSufS-HA
days +ATc
kDa
4
0
1
2
3
4
100
70
55
25
Lipoylated TgPDH-E2
TgCPN60
TgSAG1
B
Mitochondrial respiratory chain
DNA repair and replication
Basal complex constriction
Catabolism/peptidase
Ribosome biogenesis
Amino acid metabolism
RNA-interacting/regulating
SRS
Unknown
0
10
20
30
40
50
% of candidates with decreased expression in TgSufS mutant
Plastid protein import
RNA-interacting/regulating
Organophosphorus synthesis
NAD/NADP synthesis
Sugar metabolism
Pentose phosphate pathway
Receptor
Folate metabolism
SRS
Catabolism/peptidase
Channel/transporter
Lipid metabolism
GRA
Unknown
0
10
20
30
40
% of candidates with increased expression in TgSufS mutant


A
Decreased expression in TgIscU mutant
Micronemes
ER
Dense granules
Apicoplast
N/A
PM
Cytosol
Nucleus
Mitochondrion
Increased expression in TgIscU mutant
Proteasome
Cytoskeleton
Rhoptry
ER
Micronemes
Apicoplast
Golgi
VAC/endomembrane system
Mitochondrion
Nucleus
N/A
PM
Cytosol
Dense granules
B
Fe-S cluster biosynthesis
Regulation of iron and ROS
DNA repair and replication
Stress response
Mitochondrial protein import
Channel/transporter
Isoprenoid metabolism
GRA
Ribosome biogenesis
Catabolism/peptidase
Amino acid metabolism
SRS
RNA-interacting/regulating
Unknown
Mitochondrial respiratory chain
0
10
20
30
40
% of candidates with decreased expression in TgIscU mutant
Mitochondrial respiratory chain
NAD/NADP synthesis
Organophosphorus synthesis
CoA synthesis
Lipid metabolism
Nucleic acid synthesis
Stress response
Glycolysis/gluconeogenesis
Amino acid metabolism
Oxidative stress response
RNA-interacting/regulating
Pentose phosphate pathway
Catabolism/peptidase
Folate metabolism
Channel/transporter
SRS
GRA
Unknown
0
10
20
30
% of candidates with increased expression in TgIscU mutant


A
TATi ΔKu80
96h ATc
cKD TgIscU-HA
96h ATc
B
no JC-1
no ATc
+ATc
48h
96h
P1
100,0
P2
0,040
P3
0
P1
4,31
P2
15,9
P3
79,2
P1
2,80
P2
23,5
P3
73,7
P1
3,82
P2
35,3
P3
61,0
TATi ΔKu80
P1
9,78
P2
26,0
P3
64,3
P1
20,8
P2
53,4
P3
25,9
P1
19,7
P2
47,0
P3
33,5
cKD TgIscU-HA
Side scatter
(cells)
Fluorescence intensity
(red)


A
DBL IMC3 DAPI
24h
48h
72h
96h
120h
B
Percentage of DBL-positive vacuoles
35%
30%
25%
20%
15%
10%
5%
0%
*
*
24h
48h
TATi ΔKu80+ATc
TATi ΔKu80+100µM NO
cKD TgIscU-HA-ATc
cKD TgIscU-HA+ATc
cKD TgSufS-HA-ATc
cKD TgSufS-HA+ATc
C
log2-transformed fold change
-3 -2 -1 0 1 2 3 4
Bz/Tz
TgIscU/Ctrl
TgSufS/Ctrl
Bz-enriched
Tz-enriched
TGME49_320230 SRS15C
TGME49_320190 SRS16B
TGME49_273110 SRS30D
TGME49_207140 SRS49B
TGME49_222370 SRS13
TGME49_264660 SRS44
TGME49_320180 SRS16C
TGME49_315410 SRS53F
TGME49_292280 SRS36D
TGME49_320170 SRS16E
TGME49_321480 SRS12B
TGME49_226860 SRS67
TGME49_301180 SRS19F
TGME49_315320 SRS52A
TGME49_273130 SRS30A
TGME49_208850 SRS11
TGME49_267140 SRS38B
TGME49_271050 SRS34A
TGME49_315740 SRS54
TGME49_233480 SRS29C
TGME49_233460 SRS29B
TGME49_233450 SRS29A
D
SAG1
P18/SAG4
P21
No ATc
+ATc 7 days
+ATc 20 days
E
Cyst area (µm2)
1500
1000
500
0
****
+ ATc 7 days
+ ATc 20 days