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Divergent cytochrome c maturation system in kinetoplastid protists

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Running head: kinetoplastid cytochrome c maturation

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**ABSTRACT** In eukaryotes, heme attachment through two thioether bonds to mitochondrial cytochromes c and c\textsubscript{1} is catalysed by either multi-subunit cytochrome c maturation system I or holocytochrome c synthetase (HCCS). The former was inherited from the \(\alpha\)-proteobacterial progenitor of mitochondria; the latter is a eukaryotic innovation for which prokaryotic ancestry is not evident. HCCS provides one of few exemplars of \textit{de novo} protein innovation in eukaryotes, but structure-function insight of HCCS is limited. Uniquely,
euglenozoan protists, which include medically relevant kinetoplastids *Trypanosoma* and *Leishmania* parasites, attach heme to mitochondrial c-type cytochromes by a single thioether linkage. Yet the mechanism is unknown as genes encoding proteins with detectable similarity to any involved in cytochrome c maturation in other taxa are absent. Here, a bioinformatics search for proteins conserved in all hemoprotein-containing kinetoplastids identified kinetoplastid cytochrome c synthetase (KCCS), which we reveal as essential, mitochondrial, and catalyses heme attachment to trypanosome cytochrome c. KCCS has no sequence identity to other proteins, apart from slight resemblance within four short motifs suggesting relatedness to HCCS. Thus, KCCS provides a novel resource for studying eukaryotic cytochrome c maturation, possibly with wider relevance since mutations in human HCCS leads to disease. Moreover, many examples of mitochondrial biochemistry are different in euglenozoans as compared to many other eukaryotes; identification of KCCS thus, provides another exemplar of extreme, unusual mitochondrial biochemistry in an evolutionarily divergent group of protists.

**IMPORTANCE** Cytochromes c are essential proteins for respiratory and photosynthetic electron transfer. They are post-translationally modified by covalent attachment of a heme cofactor. Kinetoplastids include important tropical disease-causing parasites; many aspects of their biology differ from other organisms, including their mammalian or plant hosts. Uniquely, kinetoplastids produce cytochromes c with a type of heme attachment not seen elsewhere in nature and were the only cytochrome c-bearing taxa without evidence of
protein machinery to attach heme to the apo-cytochrome. Using bioinformatics, biochemistry and molecular genetics we report how kinetoplastids make their cytochromes c. Unexpectedly, they use a highly diverged version of an enzyme used for heme-protein attachment in many eukaryotes. Mutations in the human enzyme lead to genetic disease. Identification of kinetoplastid cytochrome c synthetase, thus, solves an evolutionary unknown, provides a possible target for anti-parasite drug development, and an unanticipated resource for studying the mechanistic basis of a human genetic disease.

**KEYWORDS** cytochrome c, *Leishmania*, mitochondrial metabolism, post-translational modification (PTM), protist, *Trypanosoma brucei*

Trypanosomatid parasites of the genera *Trypanosoma* and *Leishmania* are responsible for a variety of serious neglected tropical diseases and belong to the class of flagellate protists called Kinetoplastea. Numerous aspects of kinetoplastid mitochondrial biology, including genome organisation (1), RNA editing, protein/tRNA import (2) and cristae formation (3) are highly divergent or unique compared with many eukaryotes.

A fundamental aspect of mitochondrial function is covalent attachment of heme to mitochondrial cytochromes c and c₁ within the mitochondrial intermembrane space. For this post-translational modification, thioether bonds form between heme vinyl groups and cysteine sulphydryl groups of a CxxCH heme-binding motif within the apo-cytochrome. The stereochemistry of this heme attachment is conserved across evolution: the 2-vinyl group of
heme attaches to the first cysteine, the 4-vinyl group to the second, and histidine provides an axial ligand to the heme iron. In most eukaryotes, holocytochrome c synthetase (HCCS), associated with the outer leaflet of the mitochondrial inner membrane, catalyses heme attachment to mitochondrial cytochromes c (4). In the proto-mitochondrion, however, the multi-subunit, integral membrane cytochrome c maturation System I provided an ancestral pathway for c-type cytochrome biogenesis. It is retained, partially mitochondrially encoded, in many plants and a few protists. Instances of eukaryotes containing both maturation systems are extremely rare: orphan taxon and predatory flagellate Ancoracysta twista reportedly contains HCCS and System I, but is now extinct in the laboratory (5) and survey of the 1000 plant transcriptome resource (6) suggests club mosses Phylloglossum drummondii and Huperzia squarrosa possess HCCS plus nuclear-encoded System I fragments or a mitochondrial CcmF pseudogene, respectively¹.

Kinetoplastid protists are the only eukaryotes where mitochondrial cytochromes are present but evidence of a cytochrome c maturation system is absent (4, 7, 8). Moreover, kinetoplastids and other euglenozoans (e.g. Euglena gracilis), are unique in that heme is bound through only a single thioether linkage in mitochondrial cytochromes c: In Euglenozoa AAQCH and FAPCH are the conserved heme-binding motifs in cytochromes c and c₁, respectively (the residue at the proximal heme-binding cysteine in normal c-type cytochromes is underlined; the heme-binding cysteine conserved in all cytochromes c is in bold). Why euglenozoans possess mitochondrial cytochromes c with heme bound by a

¹ AB, MC, MLG, unpublished observations
single thioether bond is a mystery of almost fifty years standing. No noticeable difference in
the physicochemical properties of euglenozoan cytochromes c is known (9). Yet, the
activities of Euglena cytochrome c reductase and oxidase vary dependent upon the source
of the cytochrome c used plus there are fitness costs in trypanosomes engineered to
express only CxxCH heme-binding cytochrome c (10). This leaves it possible single cysteine
linkage affects electron transport through the mitochondrial respiratory chain (11). The
strict conservation of phenylalanine and proline within heme-binding motifs of kinetoplastid
and Euglena cytochrome c₁ is another puzzle and potentially unique. Perhaps, the proline
introduces a local bend in the polypeptide that allows accommodation of the phenylalanine
side-chain with the proteins tertiary structure (11).

To resolve how kinetoplastids mature their unique mitochondrial c-type cytochromes
we sorted candidate mitochondrial proteins to identify those conserved in all kinetoplastids,
except for plant-pathogenic Phytomonas. In Phytomonas, adaptation to carbohydrate-rich
plant latex correlates with secondary loss of mitochondrial cytochromes and other
hemoproteins (12); thus, we reasoned that in Phytomonas a cytochrome c maturation
system would also be lost. Candidate mitochondrial proteins were then screened for motifs
similar to any present in proteins belonging to the four biogenesis systems known to
catalyse heme attachment to a cysteine sulphydryl (Text S1). We identified a single
hypothetical protein, highly conserved across the Kinetoplastea (Fig. S1), but absent from
Phytomonas (encoded by Tb927.3.3890 in T. brucei; LmxM.08_29.1300 in Leishmania
mexicana) that exhibited co-linearity, but very limited sequence similarity, to four HCCS
motifs required for thioether bond formation (Fig. 1A). Similarity between this kinetoplastid
protein and human HCCS was too limited to be detected by PSI-BLAST, but the histidine (His154) essential in HCCS for heme attachment to apo-cytochrome c \( (13) \) was present at an analogous position in candidate kinetoplastid cytochrome c synthetase (KCCS).

To assess KCCS candidature, we co-expressed recombinant Tb927.3.3890 and \( T. \) brucei cytochrome c in \( E. \) coli. We reported previously \( T. \) brucei apo-cytochrome c is neither subject to spontaneous maturation in the \( E. \) coli cytoplasm nor a substrate for the endogenous periplasmic \( E. \) coli cytochrome c maturation system (which is expressed minimally under the aerobic conditions we used to cultivate our \( E. \) coli cultures) \( (7) \). Here, recombinant expression of \( T. \) brucei cytochrome c bearing an N-terminal hexa-histidine tag also resulted in no detectable holo-cytochrome c formation. Co-expression of Tb927.3.3890 and His\(_6\)-tagged \( T. \) brucei cytochrome c, however, resulted in heme attachment to the latter, as shown by SDS-PAGE of purified protein and staining for covalently bound heme \( (\text{Fig. 1B}) \). Pyridine hemochrome spectra of purified recombinant trypanosome holo-cytochrome c confirmed heme attachment via a single thioether bond \( (\text{Fig. 1C}) \): in the spectra shown, the pyridine haemochrome \( \alpha \)-band maximum of the recombinant cytochrome was 553 nm, and clearly red-shifted in comparison with the corresponding 550 nm \( \alpha \)-band maximum of cytochromes c, which bind heme via two thioether bonds (equine cytochrome c in \( \text{Fig. 1C} \)). Thus, Tb927.3.3890 is appropriately referred to as TbKCCS.

For molecular genetics analyses of KCCS, we used \( L. \) mexicana engineered for tractable CRISPR-Cas9 genome editing (denoted as T7 in \( \text{Fig. 2B} \) and T7Cas9 in \( \text{Fig. 2C-E} \)) \( (14) \). \( Leishmania \) promastigotes have no capacity for anaerobic growth; thus, mitochondrial
cytochromes are essential (15). Tagged with mNeonGreen and expressed from an endogenous chromosomal locus, LmKCCS showed mitochondrial localisation (Fig. 2A), consistent with a role in mitochondrial cytochrome c maturation. Mitochondrial localisation was observed irrespective of whether the mNeonGreen tag was N- or C-terminal providing evidence for an internal hydrophilic mitochondrial import signal, as described in yeast HCCS (16). We were unable to generate LmKCCS null mutants and were only able to delete both chromosomal copies of LmKCCS without further genome rearrangements following episomal expression of GFP-tagged LmKCCS or TbKCCS (Fig. 2B-E). In the absence of pNUS-derived episomes, CRISPR-Cas9-mediated disruption of both LmKCCS alleles in diploid L. mexicana resulted in genome duplication, as well as the site-specific integration of drug-resistance cassettes, as revealed by propidium iodide (PI) staining and flow cytometry of methanol-fixed logarithmic phase parasites (Fig. 2C). Thus, CRISPR-Cas9 genome editing indicated LmKCCS is an essential gene.

Our comparative genomics approach identified KCCS and provides a mechanism by which kinetoplastids mature their mitochondrial cytochromes c with heme attached via a single thioether bond. Other than the missing thioether bond, x-ray structures of trypanosomatid and yeast holo-cytochromes c are very similar, although the former (with either AxxCH or an engineered CxxCH heme-binding motif) is a very poor substrate for yeast HCCS (17), presumably because HCCS requires interaction with amino acids upstream of the heme-binding motif (18, 19).
Inability to detect proteins homologous to KCCS outside of the Kinetoplastea suggests KCCS is more likely to be an extreme or highly divergent HCCS, rather than derived through convergent evolution. Yet, irrespective of its origin, KCCS provides another example of 'extreme biology' within a group of protists already well-known for pushing the boundaries (20).

Further characterisation should indicate whether the amino acid differences and insertions observed in *Perkinsela* KCCS reflect this taxon's basal position in kinetoplastid phylogenies or the particular, unusual niche occupied by this obligate symbiont of *Paramoeba* (21). There is also wider significance to our observations: HCCS mutation in humans causes MLS (22, 23), but insight into HCCS catalysis, and thus the mechanistic consequence(s) of HCCS mutation, are in their infancy (13, 24-26). Our earlier work replacing ‘AxxCH’ cytochrome c in *T. brucei* with a ‘CxxCH’ variant and characterising the resultant mis-matured cytochrome (10), indicates KCCS is a highly diverged HCCS that cannot act on the first cysteine of a conventional CxxCH heme-binding motif. Indels, deletions, and substitutions within motifs I and II of KCCS, including substitution of the Glu159 MLS mutation (22), may be particularly informative for future study of HCCS catalysis since these are the motifs believed to mediate heme binding and release (13, 26). Intriguingly, Arg217, the other residue mutated in MLS patients is also not conserved in KCCS.

Methods are available as supplemental material in Text S1.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIG 1 (A) KCCS is a novel protein exhibiting very little sequence similarity to HCCS. MAFFT was used to align T. brucei (Tb) KCCS with HCCS from diverse taxa. HCCS-defining motifs I-IV are boxed; His154 is denoted by a red asterisk; mutations seen in MLS patients are indicated by blue asterisks; grey asterisks indicate residues analysed by site-directed mutagenesis of HsHCCS (10, 21); circles denote where site-directed mutation decreased HCCS activity. Cr, Chlamydomonas reinhardtii (XP_001697002.1); Hs, Homo sapiens (NP_001116080.1); Sc, Saccharomyces cerevisiae (NP_009361.1). NCBI reference sequences are provided in the parentheses. (B-C) TbKCCS-catalysed maturation of trypanosome cytochrome c (TbCYTC). (B) Soluble fractions from E. coli induced for expression (described
in Text S1) of either His$_6$-tagged $Tb$CYTC or His$_6$-tagged $Tb$CYTC plus $Tb$KCCS from pCDFDuet-1 (Novagen) were purified by Ni$_{2+}$-affinity chromatography. Flow-through (FT) and elution fractions (E6, E7) from each culture subject to acetone precipitation and analysed by SDS-PAGE under non-reducing conditions. Duplicate 12% gels were stained with either Instant Blue (to confirm protein loading) or 3,3',5,5'-tetramethylbenzidine (TMB, to detect covalent attachment of heme to protein). 50 ng of equine holocytochrome c was loaded in the control lane of both gels. Dimerization of cytochrome c, evident in all lanes is explained by ‘domain-swapping’ of the C-terminal α-helix (27, Text S1). (C) Pyridine hemochrome spectra for recombinant trypanosome (dashed line) or equine heart cytochrome c (solid line) were recorded at 25°C following disodium dithionite addition. Concentration of cytochromes analysed was 13 μM; spectra were normalised by Soret band intensity; the inset expands the 500-600 nm region of the two spectra, indicating the diagnostic α-band maximum at 553 nm for cytochrome c with heme bound by a single thioether bond, red-shifted relative to the 550 nm α-band maximum for cytochrome c with a CxxCH heme-binding motif. Instant blue- and TMB-stained gels of the purified cytochrome preparation used for spectroscopy are shown in Fig. S2.

FIG 2 (A) Mitochondrial localisation of $Lm$KCCS::mNeonGreen in live, CyGEL$^\text{TM}$-immobilized $L.\text{mexicana}$. (B-E) CRISPR-Cas9 genome editing of $L.\text{mexicana}$ reveals $Lm$KCCS is an essential gene. (B) Homologous recombination of drug-resistance cassettes into $Lm$KCCS loci with amplicons from PCR-mapping indicated. P1, amplicon within the $Lm$KCCS coding
sequence (or CDS); P2, downstream and within LmKCCS; P3, downstream of LmKCCS and within PUR or BSD resistance cassettes. Diagnostic PCR from gDNA templates extracted from *Leishmania* populations (X and Y) after transfection with sgDNA and template donor DNA for CRISPR-Cas9 gene editing. (C) Analysis of DNA content in propidium iodide-stained cell populations T7Cas9, X and Y by flow cytometry. (D) Episomal expression of LmKCCS::GFP (EC-L) with PCR amplicons for P1 and P4 or (E) episomal expression of TbKCCS::GFP (EC-T) and PCR amplicons for P5 and P6 indicated. Also shown, PCR mapping of T7Cas9 parental *L. mexicana* and FKO clones LX and LY (D) or TX and TY (E) together with immunoblot analysis of LmKCCS::GFP or TbKCCS::GFP expression in wild-type *L. mexicana*, episome-transfected *L. mexicana*, and FKO clones. For the loading control on the immunoblots, expression of oligopeptidase B (anti-OPB) was detected.

Guide to the Supplemental Material

Text S1 The complete description of methods covers (i) bioinformatics; (ii) biochemical validation of KCCS candidature (including details of plasmid construction, recombinant protein expression and purification, analysis of cytochrome c maturation by SDS-PAGE, 3,3',5,5'-tetramethylbenzidine-staining and uv/vis spectroscopy); (iii) explanation of the cytochrome c dimerization seen in Fig. 1B; (iv) genetic manipulation of *Leishmania mexicana*; (v) analysis of CRISPR-Cas9 *L. mexicana* mutants by flow cytometry; and (vi) analysis of KCCS localisation by fluorescence microscopy.
Supplemental Figure legends

**FIG S1** KCCS is a conserved kinetoplastid protein. Ad, *Angomonas deanei* (EPY32355.1); Bs, *Bodo saltans* (CUF09763.1); Lm, *Leishmania mexicana* (XP_003872427.1); Pk_sp, *Perkinsela* (KNH04224.1); Tb, *Trypanosoma brucei* (XP_843981.1); Tbr, *Trypanoplasma borrelii*.

Genbank accession numbers for the sequences used in the alignment are provided in the parentheses.

**FIG S2** Purity of and covalent heme attachment to *Tb*<sup>His</sup>CYTC purified for uv/vis spectroscopy. *Tb*<sup>His</sup>CYTC was purified from 8 l of *E. coli* induced for recombinant expression of *Tb*<sup>His</sup>CYTC and *Tb*KCCS as described in Text S1 and concentrated to 0.5 ml using a Vivaspin-20 centrifugal concentrator with a m.w. cut-off of 3 kDa. 1/250<sup>th</sup> of the purified protein was taken without acetone precipitation for analysis by SDS-PAGE under non-reducing conditions. Duplicate 12% polyacrylamide gels were stained with either Instant Blue (to confirm purity) or 3,3′,5,5′-tetramethylbenzidine (to detect covalent attachment of heme to protein). Equine holocytochrome c was loaded as indicated.
Extreme divergence of the kinetoplastid cytochrome c maturation system

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TEXT S1 Materials and Methods

Bioinformatics. KCCS was identified from a bioinformatics sift of proteins present in T. brucei mitochondrial proteomes (1, 2). 295 proteins identified as without orthologues in Phytomonas were then analysed manually for presence of any possible motifs or similarity to motifs present in any protein previously characterised as involved in any of the four biogenesis systems known to catalyse thioether bond formation between heme and a cysteine sulphhydril (3-6). Disorder predictions were made using IUPredA and MFDp2 (7, 8). Multiple sequence alignment (MAFFT) was used to align peptide sequences (9).

Biochemical validation of KCCS candidature. For expression of recombinant cytoplasmic TbHCCS and/or TbCYTC, CDSs were sub-cloned into pCDFDuet-1 (Novagen): TbCYTC was sub-cloned into HindIII-EcoRI-digested multiple cloning site 1 (or MCS1), TbHCCS was sub-cloned into XhoI-NdeI-digested multiple cloning site 2 (or MCS2). Forward and reverse primer combinations for PCR amplification of TbHCCS or TbCYTC, CDSs, respectively, from genomic DNA templates were ttgaattcgtgcatgccccaaaggagcgtgc and gcagaagcttttagctctttataatgctcagg or caccatatgtaggtgggtaggacattcctgc and aagctcagtcacccgtagctcagctcattttac. Restriction sites introduced into the primers are italicised. Recombinant protein expression of TbHCCS, His\(_6\)-TbCYTC, or TbHCCS and His\(_6\)-TbCYTC was induced in E. coli Rosetta (Novagen) by addition of 1mM IPTG; induced cultures
were allowed to grow for 24 h at 18°C at 100 rpm under aerated conditions. *E. coli* cultures were grown in Luria broth (Melford L24400-500.0) without addition of exogenous heme. Following induction of recombinant protein expression, bacterial cells were collected by centrifugation and re-suspended in 20 ml lysis buffer (Tris-HCl (20 mM pH 8.0); NaCl (500 mM); Triton X-100 (0.02% v/v); imidazole (20 mM); glycerol (10% v/v)) per l culture. Protease inhibitor PMSF (100 mM) was immediately added (10 μl per ml of resuspended cells) and the resuspension left shaking (50 rpm, room temperature, 30 min). After this incubation, lysing cells were subject to further disruption by ultra-sonication using a burst frequency of 5 sec on/15 sec off for 10 min at an amplitude of 85%. Following sonication, the suspension was centrifuged at 15 000 x g (30 min; 4°C) and the supernatant stored at -20°C prior to protein purification. Apo- and holocytochromes c were purified by Ni²⁺-affinity chromatography using Amintra Ni-NTA resin (1 ml per l culture harvested) under native conditions using wash (Tris-HCl (20 mM pH 8.0); NaCl (300 mM); Triton X-100 (0.02% v/v); imidazole (20 mM); glycerol (10% v/v)) and elution (Tris-HCl (20 mM pH 8.0); NaCl (300 mM); Triton X-100 (0.02% v/v); imidazole (500 mM); glycerol (10% v/v)) buffers. Purified cytochromes were concentrated using a Vivaspin-20 centrifugal concentrator with a m.w. cut-off of 3 kDa Prior to SDS-PAGE proteins were typically subject to acetone precipitation; SDS-PAGE was carried out under non-reducing conditions; and prior to gel-loading samples were heated to 95°C for 5 min in loading buffer containing SDS (2%); glycerol (10%); bromophenol blue (0.01%); and Tris-HCl (100 mM pH 6.5). Heme-staining of SDS-PAGE gels using 3,3',5,5'-tetramethylbenzidine (TMB) was carried out as described previously (10). Pyridine hemochrome spectra were acquired using a Cary 4000 uv/vis spectrophotometer
following the protocol laid out by Barr and Guo (11). In our experiments purified
recombinant TbCYTC or horse heart cytochrome c were soluble in 50 mM Tris-HCl (pH 7.5).

To measure oxidized heme spectra, protein solutions were mixed 1:1 with a solution of
NaOH (0.2 M), pyridine (40% v/v) and K$_3$Fe(CN)$_6$ (500 μM) in a final volume of 1 ml; to
subsequently measure reduced heme spectra, 10 μl of Na$_2$O$_4$S$_2$ (0.5 M) in 0.5 M NaOH was
added to protein/pyridine/ K$_3$Fe(CN)$_6$ solutions. Difference (reduced minus oxidized) spectra
are shown in Fig. 1C.

**Explanation of cytochrome c dimers.** From Fig. 1B: oligomerization of mitochondrial
cytochrome c has been known for almost 60 years (12) and is evident in cytochrome c
preparations from the 1940s (13). Dimerization results from displacement of the C-terminal
α-helix from monomeric cytochrome c and replaced by the corresponding (also displaced)
helix from another cytochrome c molecule (14). This readily explains the dimerization
evident in all lanes where cytochrome c is present in the Instant Blue-stained gel from Fig.
1B albeit that there is a large proportion of dimer relative to monomer purified from *E. coli*
expressing either *Tb*cytc or *Tb*cytc plus *Tb*KCCS. Where *Tb*KCCS is expressed simultaneously
with its apo-cytochrome substrate, comparison of TMB- and Instant Blue-stained gels
indicates only a proportion of trypanosome cytochrome is present in the holo-state (note
the intensity of the TMB stain for equine cytochrome c relative to the amount of protein
loaded). This we ascribe to the absence of additional, exogenous heme from the LB medium
used to culture our *E. coli* and/or low expression of soluble *Tb*KCCS relative to apo-*Tb*cytc
from the pCDFDuet-1 expression plasmid. Potentially, an extremely faint detection of heme
is detected in lane E6 for *E. coli* expressing TbCYTC but not TbKCCS; we suggest if TMB-
staining is present it possibly reflects coordination of heme iron by the N-terminal hexa-
histidine tag in the recombinant trypanosome cytochrome c.

**Cell culture and transfection.** *L. mexicana* (M379-T7Cas9) promastigotes were grown in
HOMEM medium (Gibco) supplemented with 10 % heat inactivated fetal calf serum (Gibco)
with 1 % Penicillin/Streptomycin (Sigma-Aldrich) at 25°C. Transfections were carried out
using the 4D Nucleofactor™ (Lonza).

Gene deletion studies of *Lm*KCCS were carried out using a CRISPR-Cas9 toolkit in *L.
mexicana* promastigotes genetically modified for constitutive expression of Cas9 nuclease
and T7 RNA polymerase (15). This was done by providing resistance cassettes using primers
with homology sites of 30 nucleotides upstream (F:
ACGTCGATTCGACGACTCCACAAGGAGAgtataatgcagacctgctgc) and downstream (R:
CTTGGCCAGCGCTGCAGAAAGGGAAAGCGGccaatatttgagagacctgtgc) of the break site and
were amplified from pPLOT plasmids. Guide DNA primers were used as a template for the
sgRNA induced break site (5’ gaatattaacgactcactataggGGCGGTAATTGTGGCGGCAG
gtttagagctgtagaatgc and 3’ gaatattaacgactcactataggAGCGGTACCACACGAGC"
81
gtttagagctgtagaatgc).

pNUS-GFPcN (16) was used for episomal expression of *Lm*KCCS or *Tb*HCCS, C-
terminally tagged with GFP. For cloning *Lm*KCCS, the fragment was amplified using forward
(cacttgcaagcgaattcctatagATGGCGGGGGCGGCTTC) and reverse primer
gctcatttgactcattagCGAGTGAGGACATTCCTG) and reverse primer.
For cloning *Tb*HCCS, the fragment
was amplified using forward (cacttgcaagcgaattcctatagATGTGGGTGAGGACATTCCTG) and
reverse primer (gctcatggtaccagatctatatgCGGTGCCGATGGCATTT). The PCR products were cloned into pNUS-GFPcN using Gibson assembly (NEB) as per manufacturer’s instructions. Following confirmation of episomal LmKCCS::GFP (EC-L in Fig. 2D) or TbKCCS::GFP (EC-T in Fig. 2E) expression, CRISPR-Cas9 was again used for gene deletion of LmKCCS. PCR was used as a diagnostic tool to confirm facilitated knock out (FKO) using the following primer sets P1, (forward primer) TGGGCAGCGAGTTCAAGAT and (reverse primer) AAGCGGGCAGAACTTCATCA (amplicon size 0.62 kb); P2, TGTCATTGTGACAGTGC and AAGCGGGCAGAACTTCATCA (1.027 kb); P3, TGTCATTGTGACAGTGC and GCAGCAGGTCTGCATTATA (0.178 kb); P4, TGGGCAGCGAGTTCAAGAT and GCATCACCTTCACCCTCTCC (1.025 kb); P5, CGGGTGCAAATCACCCAATG and CCTCCGAGTGTCATCCATCG (0.294 kb); P6, CGGGTGCAAATCACCCAATG and GCATCACCTTCACCCTCTCC (1.088 kb). As the protein loading control for immunoblot analysis of LmKCCS::GFP or TbKCCS::GFP expression, polyclonal antibodies detecting oligopeptidase B (anti-OPB) (17) were used.

Flow cytometry. Logarithmic cells (~5x10^6-10^7 cells ml^-1) were washed in PBS and resuspend in 70 % methanol for 30 minutes. Cell were pelleted and washed with PBS containing 10 µgml^-1 propidium iodide and 10 µgml^-1 RNAase A. Fluorescence was measured using the PE-Cy5-Lin channel on Cyan and analyses carried using Summit V4 software (Beckman Coulter). Gating included all singlet cells.

KCCS localisation. Live imaging of promastigotes expressing LmKCCS::mNeonGreen was carried out using CyGEL™ (Biostatus) to immobilize cells. Samples were imaged
immediately using a Zeiss AxioObserver microscope with 488 and 405 nm lasers. Images were processed using Zen Black (Zeiss) and Microvolution™ deconvolution software.

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FIG S1  KCCS is a conserved kinetoplastid protein. Ad, *Angomonas deanei* (EPY32355.1); Bs, *Bodo saltans* (CUF09763.1); Lm, *Leishmania mexicana* (XP_003872427.1); Pk_sp, *Perkinsela* (KNH04224.1); Tb, *Trypanosoma brucei* (XP_843981.1); Tbrr, *Trypanoplasma borreli* (). Genbank accession numbers for the sequences used in the alignment are provided in the parentheses.
Extreme divergence of the kinetoplastid cytochrome c maturation system
Asma Belbelazi, Rachel Neish, Martin Carr, Jeremy C. Mottram, and Michael L. Ginger

FIG S2 Purity of and covalent heme attachment to Tb\textsuperscript{His}CYTC purified for uv/vis spectroscopy.

Tb\textsuperscript{His}CYTC was purified from 8 l of E. coli induced for recombinant expression of Tb\textsuperscript{His}CYTC and TbKCCS as described in Text S1 and concentrated to 0.5 ml using a Vivaspin-20 centrifugal concentrator with a m.w. cut-off of 3 kDa. 1/250\textsuperscript{th} of the purified protein was taken without acetone precipitation for analysis by SDS-PAGE under non-reducing conditions. Duplicate 12% polyacrylamide gels were stained with either Instant Blue (to confirm purity) or 3,3',5,5'-tetramethylbenzidine (to detect covalent attachment of heme to protein). Equine holocytochrome c was loaded as indicated.