JEM

SUPPLEMENTAL MATERIAL

Coppi et al., http://www.jem.org/cgi/content/full/jem.20101488/DC1

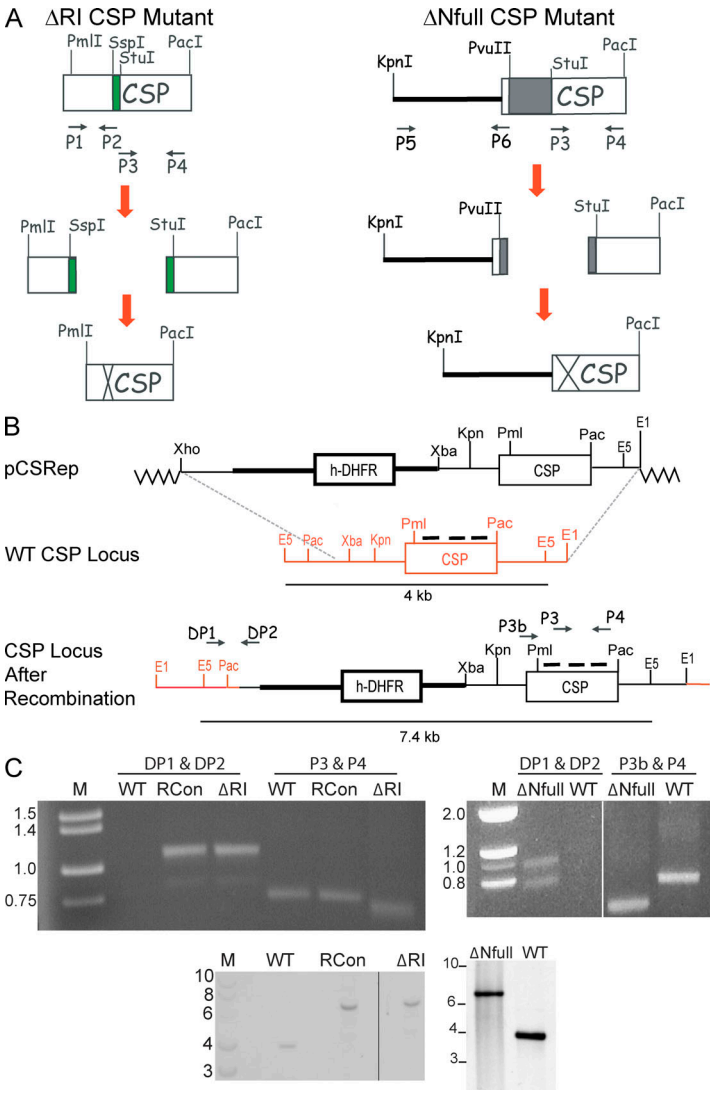


Figure S1. Construction and verification of *CSP* mutant parasites. (A) Construction of mutant *CSP* genes. A PCR-based strategy was used to make two different deletions in the *CSP* gene. In each case, two gene fragments flanking the region to be deleted and including engineered or endogenous restriction sites were amplified and cut to yield fragments that when ligated made a *CSP* mutant containing the desired deletion. The left panel shows the strategy for ΔRI *CSP* construction, and the right panel shows the strategy for ΔNfull *CSP* construction. For both mutants, primers P3 and P4 were used to amplify the 776-bp gene fragment containing the repeat region and the 3′ portion of *CSP*. For ΔRI *CSP*, the 5′ gene fragment was generated using primers P1 and P2 containing a new SspI site. For the ΔNfull CSP mutant, primers P5 and P6 were used to amplify a 5′ product containing an engineered PvuII site. Each PCR product was digested with the indicated restriction enzymes, which resulted in the deletion of the bases corresponding to the amino acid sequences KLKQP for ΔRI *CSP* and NKSIQAQRNLNELCYNEGNDNKLYHVLNSKNGKIYNRNTVNRLLADAPEGKKNEKKNEKIERNNKLKQP for ΔNfull *CSP*. A blunt end ligation was performed, and the product was sequenced and then cloned into pCSRep. Shown at the top of each panel is the full-length *CSP* gene (box) with or without a portion of its 5′ UTR (thick black line). The deleted part of *CSP* is shown in green (ΔRI) or gray (ΔNfull), and the arrows indicate the location of the primers used to generate each mutant. Following this are the PCR fragments used to generate each mutant, followed by a diagram of the resulting *CSP* gene after restriction digestion and ligation, with an X marking the location of the deleted sequence. (B) Strategy used to replace the endogenous *CSP* locus with WT or mutant *CSP*. The transfection plasmid (pCSRep) contains 730 bp of *CSP* 5′ UTR (thin black line), the selectable marker *hDHFR* with its upstream and downstream control elements (thick black lines), and the *CSP* gene flanked by its upstream and downstream control elements (thin black lines). The dotted gray lines indicate the location of homologous recombination with the endogenous locus (WT CSP Locus). Thick dashed lines above the *CSP* gene show the area of homology with the probe used in the Southern blot. The size of the fragments in the endogenous and recombined loci expected to hybridize with the probe is shown below each respective locus. DP1 and DP2 show the positions of the primers used for diagnostic PCR, and P3 (or P3b) and P4 show positions of the primers used to amplify and sequence *CSP* after recombination. E5, EcoRV; E1, EcoRI. (C) Verification of *CSP* mutant clones by PCR and Southern blot. PCR with primers DP1 and DP2 was used to verify integration into the correct genomic locus for RCon and ΔRI clones (top left) and ΔNfull clone (top right; thin white line indicates that these PCRs were run on separate gels, which are shown here aligned to the molecular weight markers on the left). PCRs with primers P3 and P4 (for RCon and ΔRI clones) or primers P3b and P4 (for ΔNfull clones) were used to amplify the *CSP* gene, which was then sequenced to confirm the presence of the deletion (not depicted). Southern blots (bottom) of EcoRV-digested genomic DNA probed with the indicated fragment of *CSP* showing the expected 4-kb band in WT parasites and 7.4-kb band in recombinant parasites. The black vertical line indicates that intervening lanes have been spliced out.

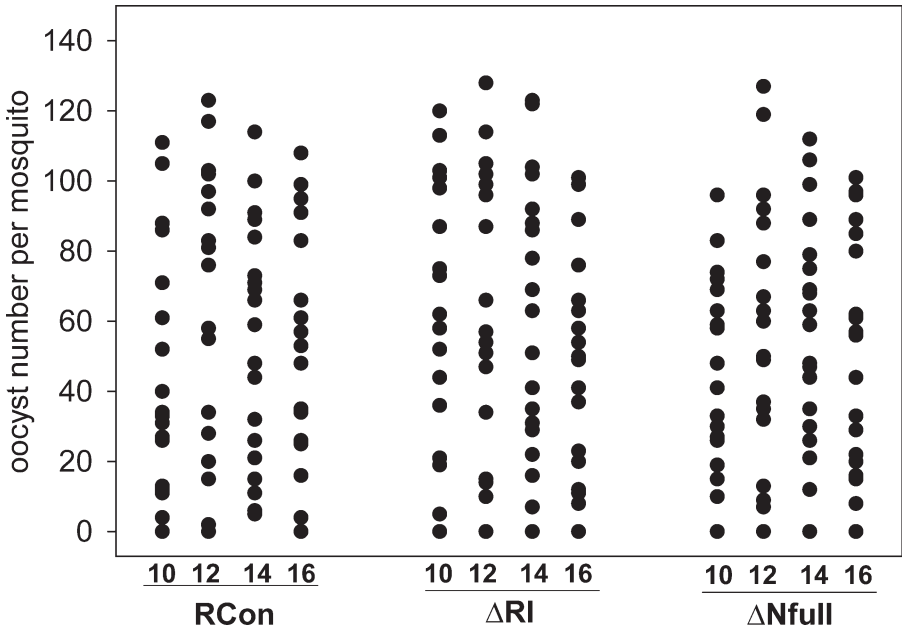


Figure S2. Oocyst numbers in *CSP* mutant parasites. Mosquitoes infected with the indicated mutants were dissected on days 10, 12, 14, and 16 after infective blood meal, their midguts were placed on a glass slide, and oocysts were counted by phase microscopy. Shown is the number of oocysts associated with each of the 20 midguts counted per time point. This experiment was performed twice with similar results.

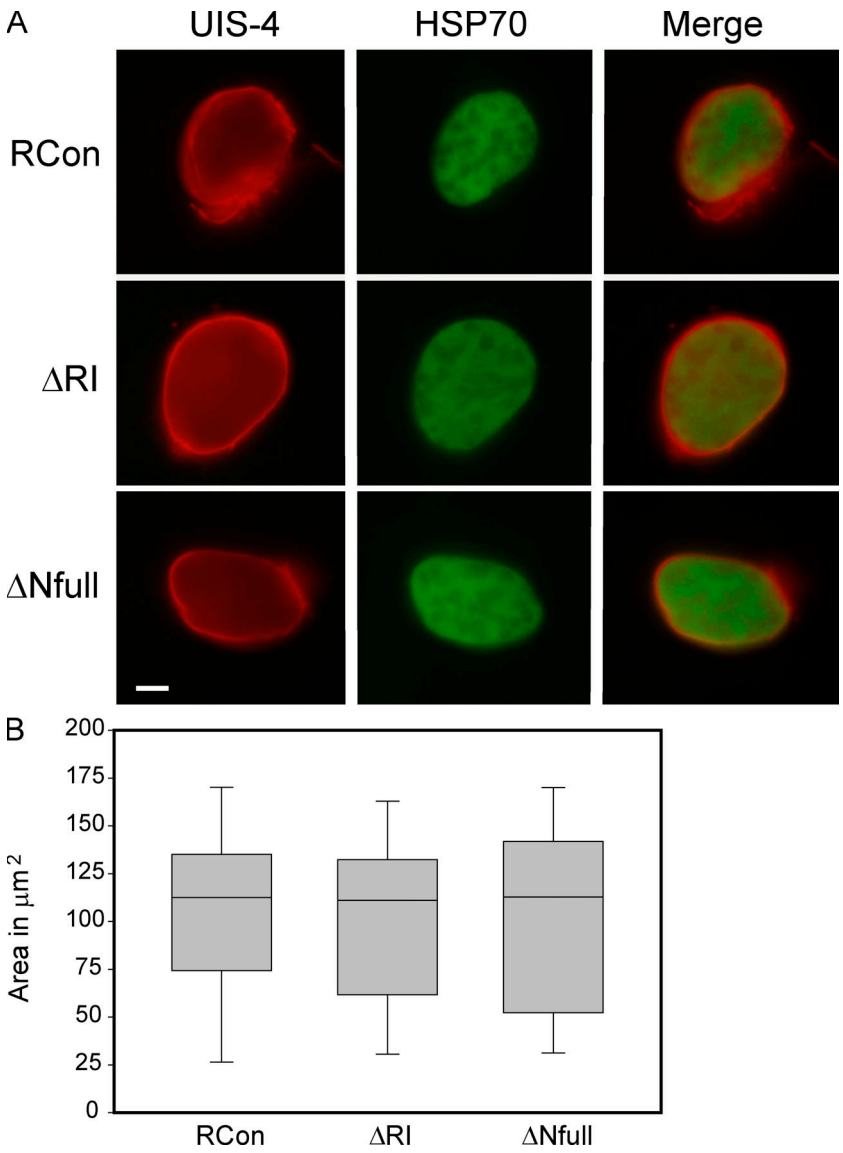


Figure S3. EEF size of *CSP* mutant parasites. (A) Fluorescence images of EEFs. 48 h EEFs were stained with antiserum to UIS-4 (red) and Hsp70 (green). Shown is a representative photo of each mutant. Bar, 5 µm. (B) Size of EEFs. Box plot graph in which the line within each box indicates the median size, box boundaries show the 25th and 75th percentiles, and error bars show the 90th and 10th percentiles. 20 EEFs per mutant line were measured using ImageJ software (National Institutes of Health). This experiment was performed twice with similar results.

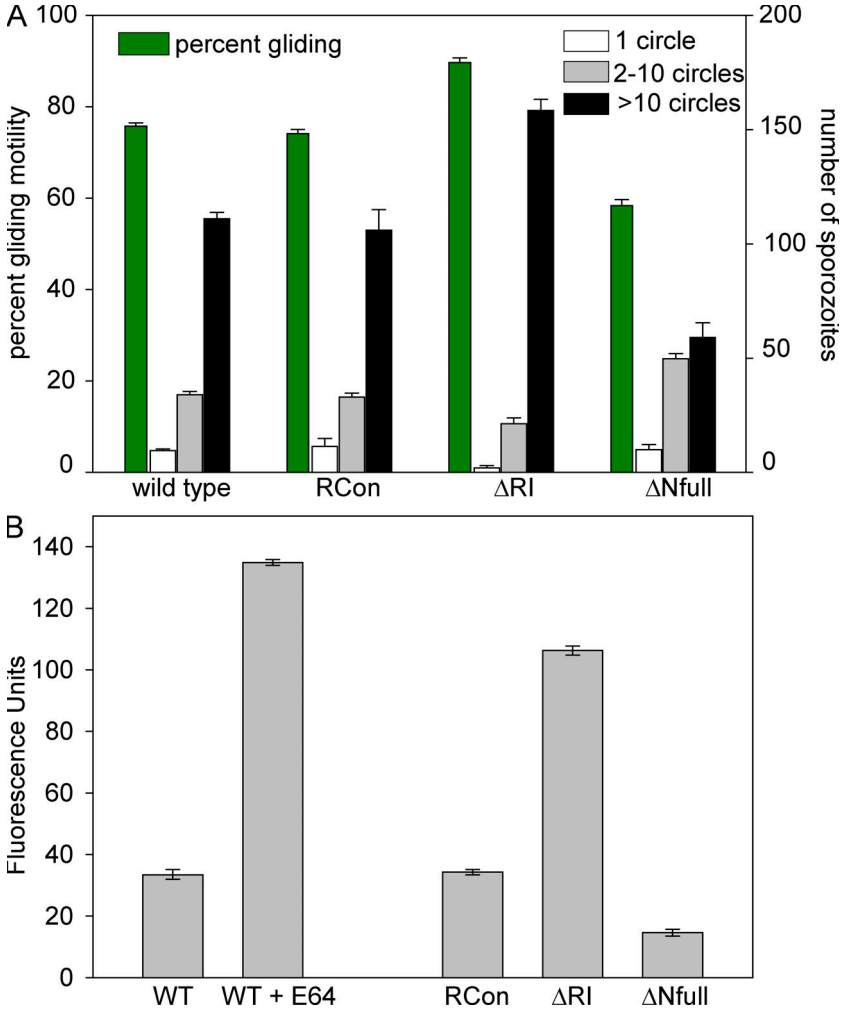


Figure S4. Gliding motility and cell traversal of *CSP* mutant parasites. (A) Gliding motility. WT, RCon, ΔRI, and ΔNfull sporozoites were added to Lab-Tek wells precoated with mAb 3D11. After 1 h at 37°C, wells were fixed and stained to visualize sporozoites and their trails. Motility was quantified by counting the number of sporozoites associated with trails and, for those sporozoites with trails, counting the number of circles in each trail. Shown is the percentage of sporozoites that exhibited gliding motility and the number of these sporozoites associated with 1, 2–10, or >10 circles per trail. 200 sporozoites/well were counted, and shown are the means ± SD of triplicates. This experiment was performed three times with similar results. (B) Cell traversal. The migratory activity of ΔRI and ΔNfull sporozoites was evaluated using the calcein assay as previously described (Coppi et al., 2007). In brief, Hepa1-6 cells were preloaded with calcein green, and 5×10^5 sporozoites pretreated with or without 10 µm E-64d for 15 min were added to cells for 1 h at 37°C. Calcein green released into the supernatant by migrating sporozoites was measured using a Fluoroskan II (Labsystems). Shown are the means ± SD of duplicate values. This experiment was performed twice with similar results.

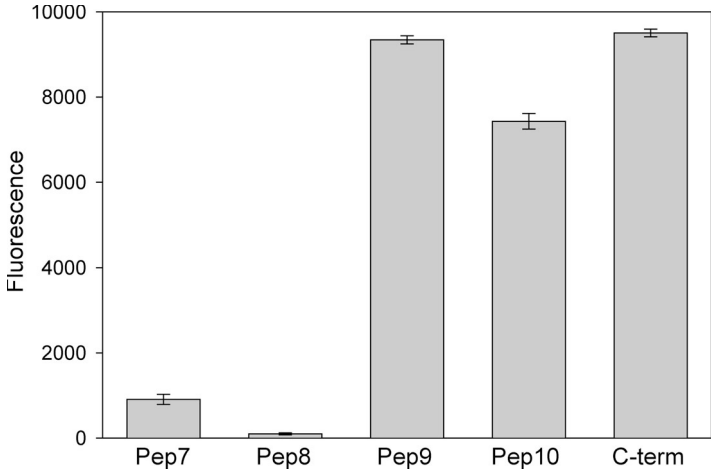


Figure S5. Fine specificity of C–terminal antiserum. Peptides were coated onto wells of microtiter plates. After overnight incubation, plates were blocked, and a 1:100 dilution of the C-terminal antiserum was added. Binding of antisera was revealed with anti–rabbit Ig conjugated to alkaline phosphatase followed by the fluorescent substrate 4-methylumbelliferyl phosphate. Fluorescence was read in a Fluoroskan II plate reader. Shown are the means of triplicates ± SD. Sequences of the peptides are Pep7, NDDSYIPSAEKILEFVKQI; Pep 8, FVKQIRDSITEEWSQCNVT; Pep 9, QCNVTCGSGIRVRKRK GSNKKAEDL; Pep 10, KKAEDLTLEDIDTEICKM; and C-terminal full-length peptide, NDDSYIPSAEKILEFVKQIRDSITEEWSQCNVTCGSGIRVRKRKGSNKKAEDLTLEDIDTEICKMDKCS. This experiment was repeated twice with similar results.

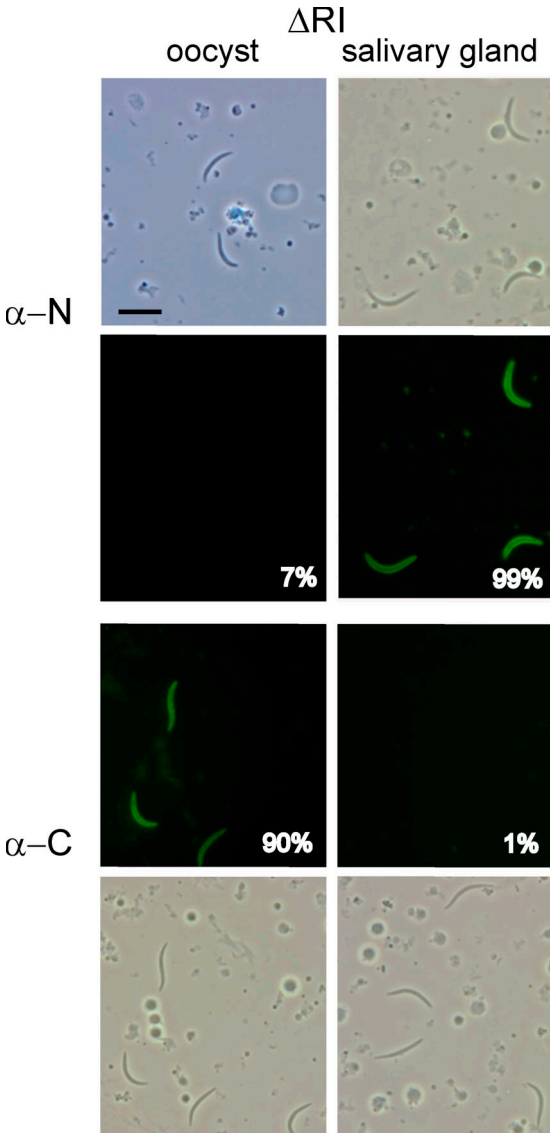
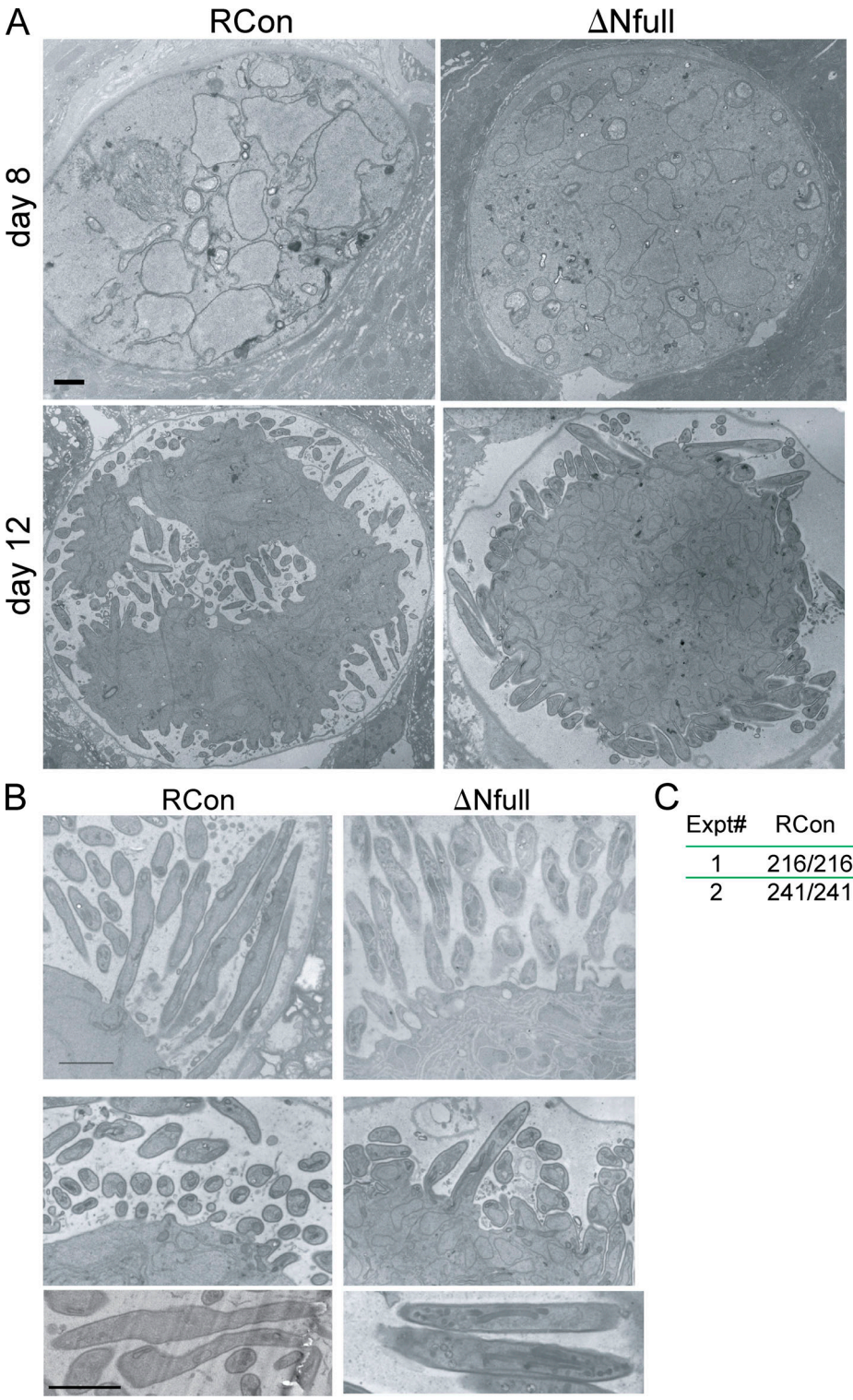


Figure S6. Surface staining of oocyst and salivary gland sporozoites of ΔRI mutants. Phase-contrast and fluorescence images of oocyst and salivary gland sporozoites from mosquitoes infected with ΔRI parasites. Sporozoites were stained with antiserum specific for the CSP N (α-N; top) or C terminus (α-C; bottom). The percentage of 200 sporozoites staining with each respective antiserum is shown on the bottom right of each fluorescence image. Bar, 10 µm.



C

Expt#	RCon	ΔNfull
1	216/216	209/209
2	241/241	253/253

Figure S7. Electron microscopy images of ΔNfull mutant oocysts. (A) ΔNfull mutant oocysts. Midguts from mosquitoes infected with the indicated parasites were dissected at days 8 and 12 after blood meal and processed for transmission electron microscopy. Photographs were taken with an EM10 transmission electron microscope. 5 oocysts from day 8 after blood meal and 8–12 oocysts from day 12 after blood meal were observed. An average of two sections per oocyst were photographed. Shown are representative images. (B) Sporozoite budding in ΔNfull oocysts. Between 8 and 12 oocysts were observed, and an average of two sections per oocyst was photographed. Shown are representative images. (C) Number of nuclei in individual RCon and ΔNfull sporozoites. Oocyst sporozoites were centrifuged onto glass coverslips, fixed with methanol, washed, and mounted in media containing 10 µg/ml DAPI. Sporozoites and their nuclei were visualized with a 100× objective using fluorescence and phase microscopy. Results are shown as number of sporozoites with one DAPI-staining nucleus over total number of sporozoites observed. Shown are the results from two independent experiments. Bars, 2 µm.

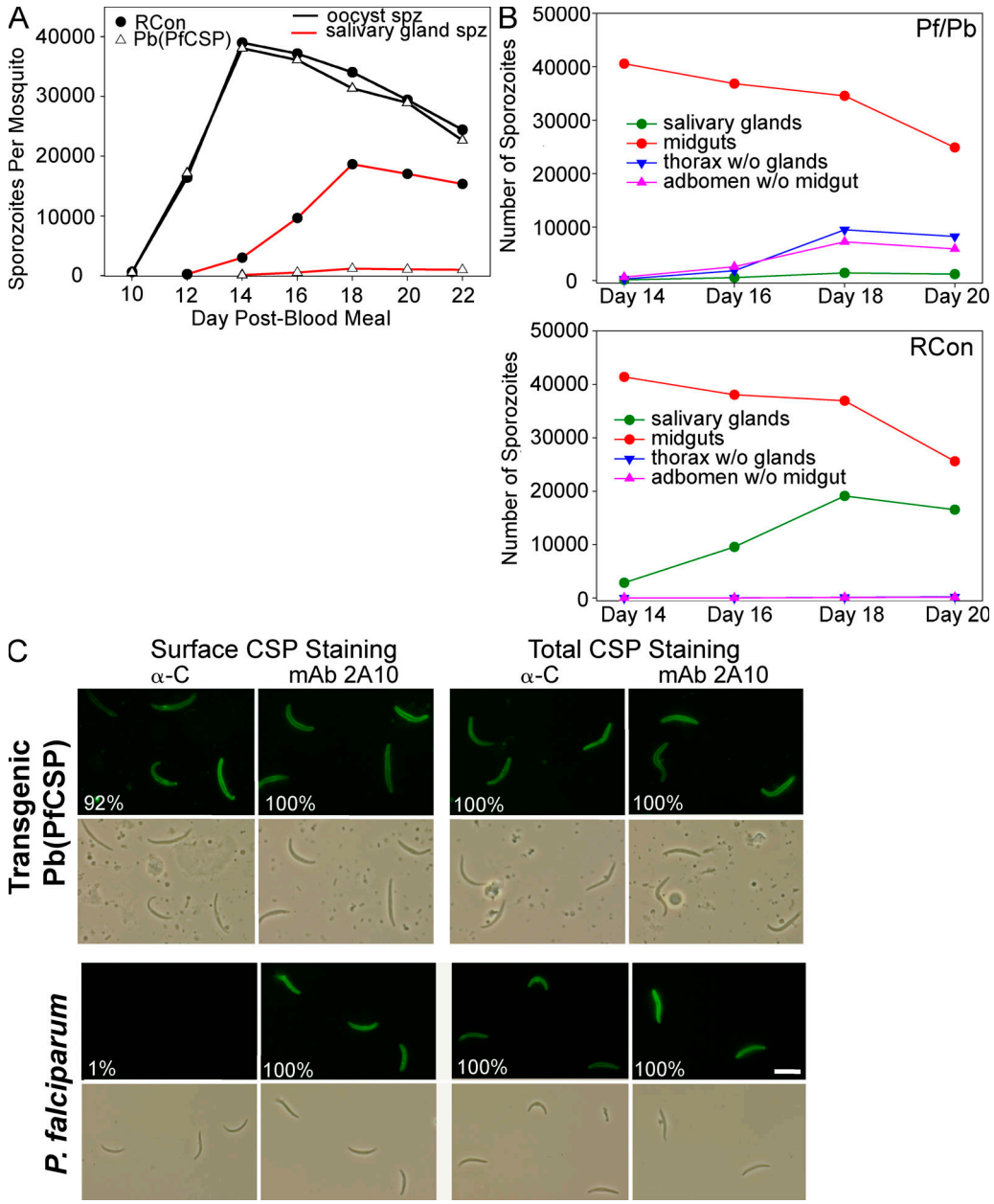


Figure S8. Mosquito distribution and immunofluorescence experiments of Pb(PfCSP) sporozoites. (A) Oocyst and salivary gland sporozoite numbers of Pb(PfCSP) parasites. Mosquitoes infected with RCon or Pb(PfCSP) parasites were dissected on the indicated days after blood meal, and the number of oocyst and salivary gland sporozoites (spz) was determined. Shown is the mean of 20 mosquitoes for each time point. This experiment was performed twice with similar results. (B) Distribution of Pb(PfCSP) sporozoites in the mosquito. Mosquitoes infected with RCon or Pb(PfCSP) parasites were dissected on the indicated days after blood meal; midguts, salivary glands, thoraces without salivary glands, and abdomens without midguts were obtained, and the total number of sporozoites associated with each was determined by RT-qPCR. Shown is the mean of 20 mosquitoes for each time point. This experiment was performed twice, and shown is a representative experiment. (C) Conformation of CSP on Pb(PfCSP) sporozoites. Fluorescence and phase-contrast images of Pb(PfCSP) and *P. falciparum* salivary gland sporozoites fixed with PFA to visualize surface staining (left) or permeabilized with methanol (right). After fixation, sporozoites were stained with either polyclonal antiserum specific for the TSR of *P. falciparum* CSP (α-C) or mAb 2A10 specific for the repeat region of *P. falciparum* CSP. The percentage of 200 sporozoites staining with each respective antiserum is shown. This experiment was performed twice with similar results. Bar, 5 µm.

Table S1. Sporozoite numbers in RCon-, ΔRI-, and ΔNfull-infected mosquitoes

Mutant and sporozoite population	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20	Day 22
RCon							
Oocyst	267 ± 34	14,911 ± 1,272	40,709 ± 1,690	33,642 ± 3,194	26,588 ± 1,386	16,259 ± 3,585	12,646 ± 2,447
Hemolymph	ND	ND	2,889 ± 159	15,268 ± 830	12,248 ± 703	9,196 ± 385	ND
Salivary gland	ND	248 ± 58	2,029 ± 121	9,057 ± 796	21,959 ± 1,203	20,063 ± 1,485	18,328 ± 1,480
ΔRI							
Oocyst	291 ± 22	15,337 ± 720	41,822 ± 4,289	36,019 ± 2,451	22,969 ± 1,563	11,083 ± 1,503	4,684 ± 845
Hemolymph	ND	ND	6,940 ± 494	15,149 ± 1,341	9,622 ± 1,041	5,443 ± 540	ND
Salivary gland	ND	154 ± 21	724 ± 77	6,651 ± 779	12,495 ± 693	11,165 ± 788	6,544 ± 777
ΔNfull							
Oocyst	816 ± 83	31,087 ± 4,789	92,633 ± 7,315	87,948 ± 6,434	71,155 ± 8,429	57,804 ± 7,202	47,142 ± 4,131
Hemolymph	ND	ND	524 ± 61	3,095 ± 281	2,165 ± 445	1,405 ± 150	ND
Salivary gland	ND	59 ± 15	304 ± 52	2,468 ± 171	4,537 ± 440	3,980 ± 553	3,459 ± 344

Mosquitoes were infected with RCon, ΔRI, or ΔNfull parasites, and at the indicated day after infective blood meal, midguts, hemolymph, and salivary glands were harvested from 20 mosquitoes and pooled, and sporozoites were counted as outlined in Materials and methods. Shown are the means of three independent experiments ± SDs.

Table S2. Localization of RCon, ΔRI, and ΔNfull sporozoites in salivary glands

Day	RCon inside/outside	ΔRI inside/outside	ΔNfull inside/outside
	%	%	%
18	79.3/20.7	76.6/23.4	66.8/33.2
19	80.8/19.2	78.5/21.5	78.1/21.9
20	80.0/20.0	78.8/21.2	72.4/27.6

Mosquito salivary glands were incubated with 50 µg/ml trypsin for 15 min at 37°C and centrifuged (80 *g*, 5 min) to pellet the glands. Sporozoites in the supernatant (outside) and salivary glands (inside) were counted, and data are expressed as the percentage of total sporozoites.

REFERENCE

Coppi, A., R. Tewari, J.R. Bishop, B.L. Bennett, R. Lawrence, J.D. Esko, O. Billker, and P. Sinnis. 2007. Heparan sulfate proteoglycans provide a signal to *Plasmodium* sporozoites to stop migrating and productively invade host cells. *Cell Host Microbe*. 2:316–327. doi:10.1016/j.chom.2007.10.002