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Supporting Information for

Knock-sideways by inducible ER retrieval enables a unique approach for studying *Plasmodium*-secreted proteins

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Supplementary Methods

Plasmids and genetic modification of *P. falciparum* and *P. berghei*. All cloning was carried out with NEBuilder HIFI DNA assembly (NEB). Primer sequences are given in Table S1. To generate NF54$^{attB\text{-}DiCre}$ parasites, a DiCre expression cassette was inserted into the *pfs47* gene in NF54^{attB} parasites (1) using a marker-free CRISPR/Cas9 editing strategy as described (2). Loss of the endogenous *pfs47* locus was monitored with primers P1/2 and P3/4 and integration was confirmed using primers P1/5 and P6/4. To confirm DiCre activity in this line, a loxP-flanked mNG coding sequence with a 3xHA tag was PCR amplified using primers P19/20 and inserted between AvrII and AflII in pLN-ENR-GFP (3), resulting in the plasmid JRB416. This plasmid was co-transfected with pINT (3) into NF54$^{attB\text{-}DiCre}$ parasites and selection was applied with 2.5 µg/ml blasticidin-S 24 hrs post-transfection.

To generate the kER fusion cassette, homology flanks targeting the 3' end of *exp2* were amplified from pyPM2GT-EXP2-3xHA-GFP11 (4) using P21/22 and inserted between XhoI and AvrII in pPM2GT-HSP101-3xFLAG (4). The loxPint sequence (5) was amplified with P23/24 and inserted immediately upstream of the 3xFLAG tag at AvrII in this vector. A partial loxPint containing the loxP sequence and 3' portion of the intron followed by a 3xHA-KDEL tag was then amplified with P25/26 and inserted into EagI, resulting in the plasmid JRB492. Finally, the mNG coding sequencing was amplified from pyPM2GT-EXP2-mNG (6) with P27/28 and inserted into AvrII in JRB492, resulting in plasmid JRB508. For generation of the *exp2* DiCre conditional knockout line, a recoded version of the *exp2* coding sequence with a 3' 3xHA tag and flanked by loxP sites was amplified using P76/77 from plasmid pEXP2^{apt} (4) and inserted between AvrII/EagI in plasmid JRB492. A 5' flank immediately upstream of the *exp2* start codon was then amplified from *P. falciparum* gDNA using P78/79 and inserted between AflII/AvrII, replacing the AflII site with AscI and resulting in the plasmid JRB467. This plasmid was linearized with AscI and co-transfected with pUF-Cas9-EXP2-CT-gRNA into NF54$^{attB\text{-}DiCre}$. Selection was applied with 5 nM WR99120 24 hrs post-transfection and clonal lines were isolated by limiting dilution after parasites returned from selection.

To generate the SP-mNG-kER reporter, the *exp2* promoter and signal peptide were amplified from parasite genomic DNA with P29/30 and the mNG-KER cassette was amplified from JRB508 with P31/32 and these amplicons were assembled together in a second PCR reaction with P29/32 and inserted into AscI/ApaI in pKD (7), resulting in the plasmid JRB543. For expression of a second copy of EXP2-kER from the attB locus, the *exp2* promoter and full-length gene were amplified from genomic DNA with P29/33 and inserted between AscI/BamHI in JRB543, resulting in the plasmid JRB526. For expression of a second copy of EXP2-kER lacking the amphipathic helix, a recoded portion of the *exp2* coding sequence beginning at codon 86 (after the amphipathic helix and immediately before the helical body domain) was amplified from pEXP2^{apt} (4) and inserted at BamHI after the EXP2 signal peptide in JRB543, resulting in the plasmid JRB544. These plasmids were each co-transfected with pINT and selected with 2.5 µg/ml blasticidin-S as described above. To relieve translational repression by TetR-DOZI, parasite cultures were supplemented with 500 nM aTc.

For generation of ClpB1-kER and RON6-kER lines, a Cas9 gRNA target was chosen just downstream (*clpB1*, ATTTTTAGAAAAATATTATG) or just upstream (*ron6*, TAATACATACAACGTACCAA) of the stop codon and the gRNA seed sequences were synthesized as primers P34 and P35, respectively, and inserted into the AflII site of pAIO3 (8), resulting in plasmids JRB143 and MAF22, respectively. To target the 3' end of *clpB1* or *ron6*, a 5' homology flank (up to but not including the stop codon) was amplified from genomic DNA using P36/37 or P38/39, incorporating synonymous shield mutations in the gRNA target sites. A 3' homology flank was amplified using P40/41 or P42/43. The corresponding 5' and 3' flank amplicons were assembled in a second PCR reaction using P40/37 and P42/39 and inserted between XhoI/AvrII in JRB508, resulting in plasmids MAF20 and MAF27, respectively. These

plasmids were linearized with AflII and co-transfected with the corresponding Cas9/gRNA plasmid into NF54$^{attB\text{-}DiCre}$. Selection was applied with 5 nM WR99120 24 hrs post-transfection and clonal lines were isolated by limiting dilution after parasites returned from selection.

For generation of PTEX150-kER and HSP101-kER lines, flank assemblies targeting the 3' end of each gene were amplified using P44/45 from pyPM2GT-PTEX150-3xHA-GFP11 (4) or P46/47 from pyPM2GT-HSP101-3xHA-GFP11 (4) and inserted between XhoI/AvrII in JRB508, resulting in plasmids MAF16 and MAF17, respectively. These plasmids were linearized with AflII and co-transfected with pAIO-PTEX150-CT-gRNA or pAIO-HSP101-CT-gRNA (4), selected with 5 nM WR99120 and cloned after parasites returned. For generation of the EXP2-kER line, plasmid JRB492 (described above) was linearized and co-transfected with pUF-Cas9-EXP2-CT-gRNA (4) and selected with 5 nM WR99120. For generation of HSP101-kER$^{TetR\text{-}DOZI}$ lines, flank assembles were amplified using P48/49 and inserted between AscI/BamHI in JRB526 (described above), resulting in plasmid MAF41. This plasmid was linearized with AflII and co-transfected with pAIO-HSP101-CT-gRNA, selected with 2.5 µg/ml blasticidin-S and 500 nM aTc, and cloned after parasites returned. For generation of HSP101-kER^{DDD} lines, the DDD was amplified from plasmid pPM2GT-HSP101-3xFlag-DDD (4) using P70/71 and inserted between AvrII/BsrGI in MAF17, replacing the mNG and resulting in plasmid MAF43. This plasmid was linearized with AflII and co-transfected with pAIO-HSP101-CT-gRNA. Selection was applied with 10 µM TMP 24 hrs post-transfection.

For expression of a second copy of PTEX150-kER from the *attB* locus, the *ptex150* promoter and full-length gene were amplified from genomic DNA with P50/51 and the mNG-KER cassette was amplified from JRB508 with P52/53. These amplicons were assembled together in a second PCR reaction using P50/53 and inserted between ApaI/AflII in pLN-ENR-GFP (3), resulting in the plasmid MAF39. This plasmid was co-transfected with pINT, selected with 2.5 µg/ml blasticidin-S and parasites were cloned after returning.

To generate the line bearing endogenous EXP2-mRuby and HSP101-mNG tags, the plasmid pbEXP2-mRuby3 (9) was first linearized with AflII and co-transfected with pUF-Cas9-EXP2-CT-gRNA (4) into NF54$^{attB\text{-}DiCre}$. Selection was applied with 2.5 µg/ml blasticidin-S. Next, the mNG coding sequence was amplified using P54/55 and inserted between AvrII/EagI in pPM2GT-HSP101-3xFLAG (4) to replace the FLAG tag with mNG, resulting in the plasmid JRB325. This plasmid was linearized with AflII and co-transfected with pAIO-HSP101-CT-gRNA (4) into the NF54$^{attB\text{-}DiCre}$::EXP2-mRuby parasites. Selection was applied with 5nM WR99120 resulting in the line NF54$^{attB\text{-}DiCre}$::EXP2-mRuby+HSP101-mNG.

For generation of PTEX150-kER and HSP101-kER lines in *P. berghei*, the plasmid pBAT (10) was digested with MluI/XhoI and the primer P56 was used as an insert to remove the intervening AvrII, SnaBI and XhoI sites from the MCS. Next, the mRuby3 coding sequence fused to the PbEXP2 signal peptide was amplified with P57/58 and inserted between SwaI/BamHI, replacing the GFP. The mNG-kER tagging sequence was amplified from plasmid JRB508 using P59/60 and inserted between SacII/SphI, replacing the mCherry-3xMYC. Finally, 5' flanks to tag *P. berghei hsp101* or *ptex150* with mNG-kER were amplified from *P. berghei* genomic DNA using P61/62 or P63/64, respectively, and 3' flanks were amplified using P65/66 or P67/68, respectively. The corresponding 5' and 3' flank amplicons were assembled with an intervening AflII site in a second PCR reaction using P65/62 or P67/64, respectively, and inserted at SacII, resulting in plasmids pPbHSP101-kER+SP-mRuby and pPbPTEX150-kER+SP-mRuby. The plasmids were linearized at AflII and transfected as described (11) into the *P. berghei* ANKA clone 2.34-based marker-free HP DiCre line (12). Transfected parasites were reinjected into naïve mice and selection was applied with 0.07 mg/ml pyrimethamine in drinking water provided *ad libitum* 24 hours later. After returning from selection, transfected populations were cloned by limiting dilution IV injection into naïve mice to generate isogenic populations designated PbHSP101-kER and PbPTEX150-kER. To monitor export with an IBIS1-mRuby fusion, the *ibis1* gene and promoter were amplified from *P. berghei* gDNA using P72/73 and the *mruby* cds was amplified using

P74/75. These amplicons were assembled in a second PCR reaction using P72/75 and inserted between PvuII/BamHI, resulting in plasmids pPbHSP101-kER+IBIS1-mRuby and pPbPTEX150-kER+IBIS1-mRuby. Plasmids were linearized at AflII and transfected as above.

P. falciparum **growth assays**. Growth assays were performed as described previously (13). Briefly, parasites were treated with either DMSO or 10 nM rapamycin for 3 hrs and washed 1X. Then, parasites were seeded between 0.2-1% parasitemia in triplicate and monitored every 48 hrs by flow cytometry on an Attune NxT (ThermoFisher) by nucleic acid staining with PBS containing 0.8 µg/ml acridine orange. For IPP rescue experiments, ClpB1-kER cultures were supplemented with 200 µM IPP. For kER fusions expressed from the *attB* locus, cultures were supplemented with 500 nM aTc 24 hrs prior to rapamycin treatment to relieve TetR-DOZI-aptamer translational repression. For HSP101-kER$^{TetR\text{-}DOZI}$ parasites, aTc was removed by washing parasites 7X with 13 mL of media. 500 nM aTc was then supplemented back to control parasites. As required, parasites were sub-cultured to avoid high parasite density, and relative parasitemia at each time point was back-calculated based on actual parasitemia multiplied by the relevant dilution factors. Data were displayed using Prism 9 (GraphPad Software).

For EC_{50} shift assays of HSP101-kER$^{TetR\text{-}DOZI}$ and HSP101-kER^{DDD}, asynchronous parasites were treated with DMSO or rapamycin before removal of aTc or TMP. After agonist washout as described above, parasites were seeded in triplicate at 0.5% parasitemia with 2-fold dilutions starting from 500 nM aTc or 250 nM TMP and parasite growth was monitored after 72 hrs.

P. berghei **kER experiments.** Parasite-infected blood was collected by cardiac puncture when parasitemia reached 3-4% and cultured in RPMI supplemented with 20% FBS. Cultures were treated with 10 nM rapamycin or a DMSO vehicle control for 3 hours followed by a media change. After 18 hours of additional culture, parasites were harvested for diagnostic PCR, live fluorescence microscopy and Western blot. Equal numbers of DMSO or rapamycin-treated infected RBCs (~10,000 parasites per injection) were IV injected into naïve mice and parasitemia was monitored by giemsa-stained blood smear every two days.

For synchronized, *ex vivo* growth assays, parasites were IP injected into mice and allowed to reach 2-3% parasitemia. Parasites gathered from cardiac puncture were then treated with DMSO or 10 nM rapamycin for 3 hrs, washed, and allowed to mature into schizonts overnight without further rapamycin treatment. The mature schizonts were magnet purified and tail vein injected into naïve mice treated with phenylhydrazine (1mg/mice) (Sigma, 114715) to allow parasites to egress and invade new RBCs in mice. After 3 hrs to allow parasite egress and re-invasion, synchronous ring-stage parasites were collected by cardiac puncture and allowed to mature overnight *in vitro*. At 24 hrs post injection, parasite development was assessed in Giemsa-stained smears and DNA content was evaluated by Hoechst 34580 staining (1:5,000) on an Attune NxT flow cytometer. To monitor export of IBIS1-mRuby, parasites were IP injected and allowed to grow to 2-3% parasitemia and imaged from tail-blood. Mice were then injected IP with rapamycin (1 mg/kg) and parasites were observed again 24hrs later.

Microscopy. For live microscopy, *P. falciparum* parasites were treated with either DMSO or 10 nM rapamycin and viewed 24-48 hrs later. Hoechst (33342, Invitrogen; 1:10,000) was used to visualize the nucleus by incubating with parasites 2-5 min prior to visualization. ER-Tracker Red (Invitrogen; 1:1000) was used to visualize the ER by incubating with parasites 30 min at 37°C prior to visualization. For constructs expressed from the *attB* locus under TetR-DOZI-aptamers control, parasites were grown in 500 nM aTc for 24 hrs prior to incubation with DMSO or rapamycin to allow expression. For RON6-kER parasites, DMSO and rapamycin-treated schizonts were incubated with 25 nM ML10 (obtained through BEI resources, NIAID, NIH and supplied by LifeArc, Stevenage, UK: MRT-0207065 (ML10), NR-56525) for 4 hrs and visualized

by microscopy. For *P. berghei*, synchronized ring-stage parasites were isolated via cardiac puncture, treated with DMSO or rapamycin for 3 hrs and allowed to develop overnight in culture before visualization. For quantification of fluorescence signal, MFI was calculated using the measured Integrated Density value from Fiji (14). The internal MFI was calculated using an ROI within the parasite PV and subtracting that from the whole parasite MFI using an ROI outside the PV boundary. The percent internal fluorescence was then calculated by dividing the internal MFI by the whole parasite MFI.

For protease protection assays on live cells, parasites were treated with 0.03% saponin (Sigma) in 1X PBS for 15 min at 4°C. Purified parasites where then washed once with 5 mL of 1X PBS and once with 5 mL of digestion buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$) (15). Parasite samples where then split into two identical tubes, one of which was treated with 20 µg/mL of Proteinase K (740506.75, Macherey-Nagel), and incubated at 37°C for 15 min. Proteinase K was removed and samples resuspended with digestion buffer plus protease inhibitor cocktail (Halt 78429, Thermo Scientific) before visualization.

For IFAs of the EXP2- and ClpB1-kER lines, parasites were treated with either DMSO or 10 nM rapamycin before fixation and processing 24 hrs or 144 hrs later as described previously (13). For HSP101-kER$^{TetR\text{-}DOZI}$ lines, parasites were synchronized with 5% sorbitol, treated with either DMSO or 10 nM rapamycin, grown with or without 500 nM aTc, and fixed 48 hrs later. Briefly, parasites were fixed with Fixative Solution (4% paraformaldehyde, 0.0075% glutaraldehyde in PBS) for 25 min at room temperature in the dark. Parasites were then permeabilized with 0.1% Triton in PBS for 10 minutes at room temperature while rocking. Parasites were washed 1X with PBS and then blocked with 3% BSA in PBS for 1 hr at room temperature. Primary antibody was applied in 3% BSA solution for 1 hr at room temperature, washed with PBS, followed by secondary antibody incubation for 1 hr at room temperature. After washing with PBS, parasites were adhered onto Poly-L-lysine coated coverslips and then mounted onto slides. For parasites probed with SBP1, parasites were acetone fixed as in (8). Briefly, thin smears were immersed in 100% acetone for 2 min. Smears were washed 1X with PBS and then blocked with 3% BSA in PBS for 1 hr at room temperature. Primary antibody was applied in 3% BSA solution for 1 hr at room temperature, washed with PBS, followed by secondary antibody incubation for 1 hr at room temperature. After washing with PBS, slides were mounted with DAPI, covered with a coverslip and sealed. The antibodies used for IFA were: mouse anti-HA antibody (clone 16B12, BioLegend; 1:200), rabbit polyclonal anti-HA SG77 (ThermoFisher; 1:500), rabbit anti-PfGRP78 (BiP) (MRA-1246, BEI Resources, NIAID, NIH; 1:100), mouse monoclonal anti-FLAG clone M2 (Sigma-Aldrich; 1:200), rabbit monoclonal anti-Flag 8H8L17 (ThermoFisher; 1:500), mouse anti-HRP2 clone 2G12 (1:500) (16), rabbit polyclonal anti-SBP1 BR28 (1:500) (17) and rabbit polyclonal anti-ACP1 (1:500 IFA) (18, 19). Secondary antibodies used were anti-mouse antibody conjugated to Alexa Fluor 488 or 546 and anti-rabbit antibody conjugated to Alexa Fluor 488, (Life Technologies; 1:1000). Parasites were mounted with ProLong diamond with 4',6'-diamidino-2-phenylindole (DAPI) (P36931; Invitrogen) and imaged on an Axio Observer 7 equipped with an Axiocam 702 mono camera and Zen 2.6 Pro software (Zeiss) using the same exposure times for all images across sample groups and experimental replicates. Image processing, analysis, and display were performed using Zeiss Blue software and Adobe Photoshop. Equivalent adjustments to brightness and contrast were made within sample groups for display purposes.

Quantification of HSP101 knockdown. To evaluate the level of HSP101 protein knockdown, HSP101-kER$^{TetR\text{-}DOZI}$ parasites were synchronized via magnet purification and treated with DMSO or rapamycin before removal of aTc. After aTc removal, parasites were seeded at ~1% parasitemia with different dilutions of aTc and allowed to grow for 48 hrs. Using an Attune NxT flow cytometer, infected RBCs were gated using Hoechst 34580 (Invitrogen; 1:5,000) and mNG

fluorescence of infected RBCs was concurrently measured. The same procedure was applied for saponin-released parasites after protease treatment as described above.

Western blotting. Western blotting was performed as described previously (4, 8). Briefly, parasite-infected RBCs were selectively permeabilized by treatment with ice-cold 0.03% saponin in PBS for 15 min followed by lysis with RIPA and centrifugation to remove hemozoin. For constructs expressed from the *attB* locus under control of TetR-DOZI-aptamers, parasites were grown in 500 nM aTc for 24 hrs prior to incubation with DMSO or rapamycin to allow for expression. For the HSP101-kER$^{TetR\text{-}DOZI}$ lines, parasites were treated with DMSO or rapamycin before removal of aTc. After aTc removal, parasites were seeded at ~1% parasitemia with different dilutions of aTc and allowed to grow for 48 hrs before processing. The antibodies used for Western blot were: mouse anti-HA antibody (clone 16B12, BioLegend; 1:500), rabbit polyclonal anti-HA SG77 (ThermoFisher; 1:500), mouse anti-FLAG (Clone M2, Sigma-Aldrich; 1:500), rabbit polyclonal anti-*Plasmodium* Aldolase ab207494 (Abcam; 1:500) and mouse monoclonal anti-EXP2 clone 7.7 (1:500) (20). The secondary antibodies used were IRDye 680CW goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG (Li-COR Biosciences; 1:20,000). Western blot images were acquired using an Odyssey Clx infrared imaging system and processed with Image Studio software (Li-COR Biosciences). Full length versions of cropped blots are shown in Figure S8.

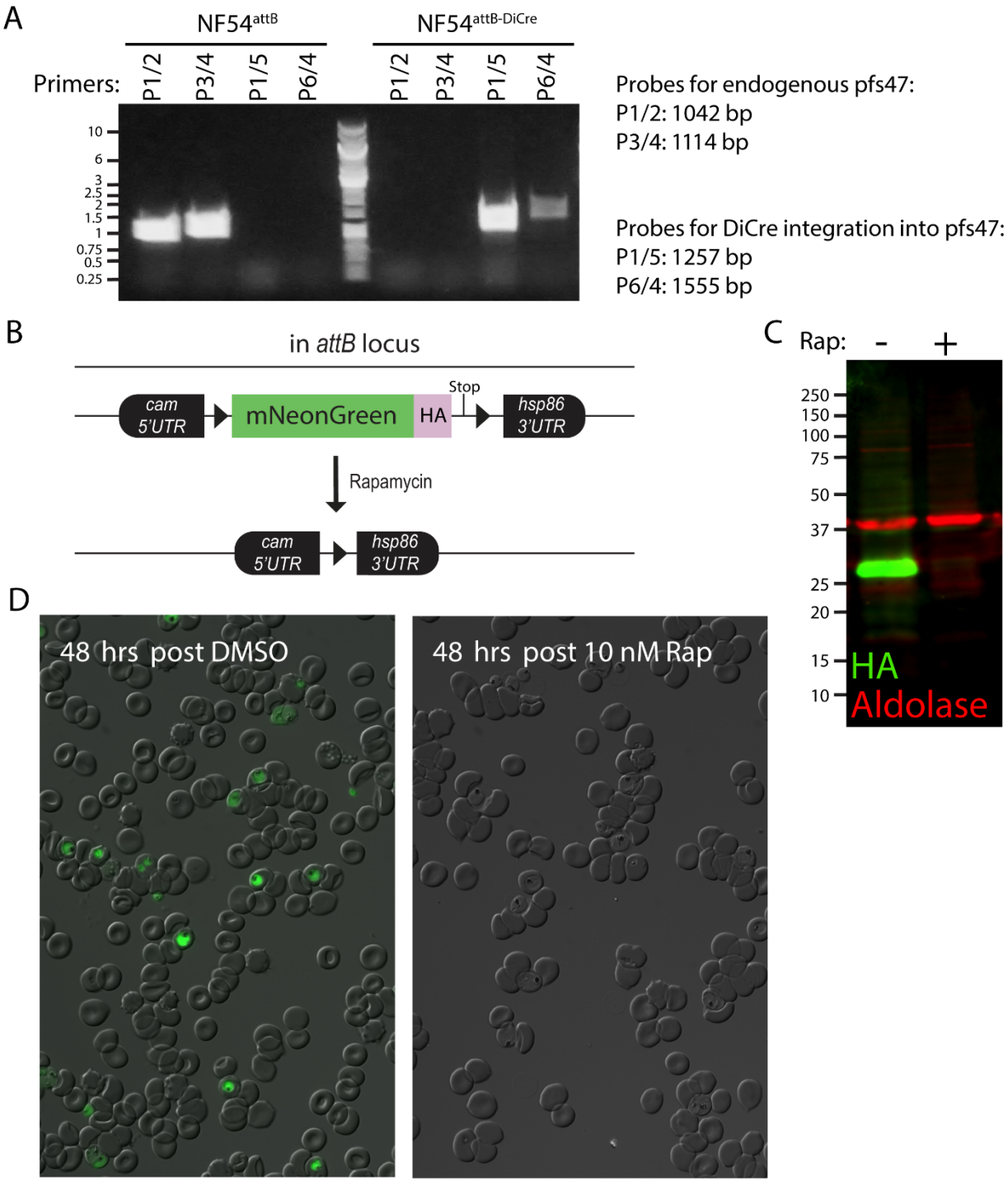

Figure S1. Generation and validation of NF54$^{attB\text{-}DiCre}$ parasites. A) Diagnostic PCR showing DiCre cassette integration into the *pfs47* locus using primers P1/2 and P3/4 to detect unmodified locus, and primers P1/5 and P6/4 to detect integration. B) Schematic of reporter integrated at *attB* in *cg6* to test DiCre functionality in NF54$^{attB\text{-}DiCre}$. C) Western blot and D) live microscopy of parasites 48 hrs after 3 hr treatment with DMSO or 10 nM rapamycin. Molecular weights are predicted to be 30 kDa for mNG-3xHA and 40.1 kDa for aldolase.

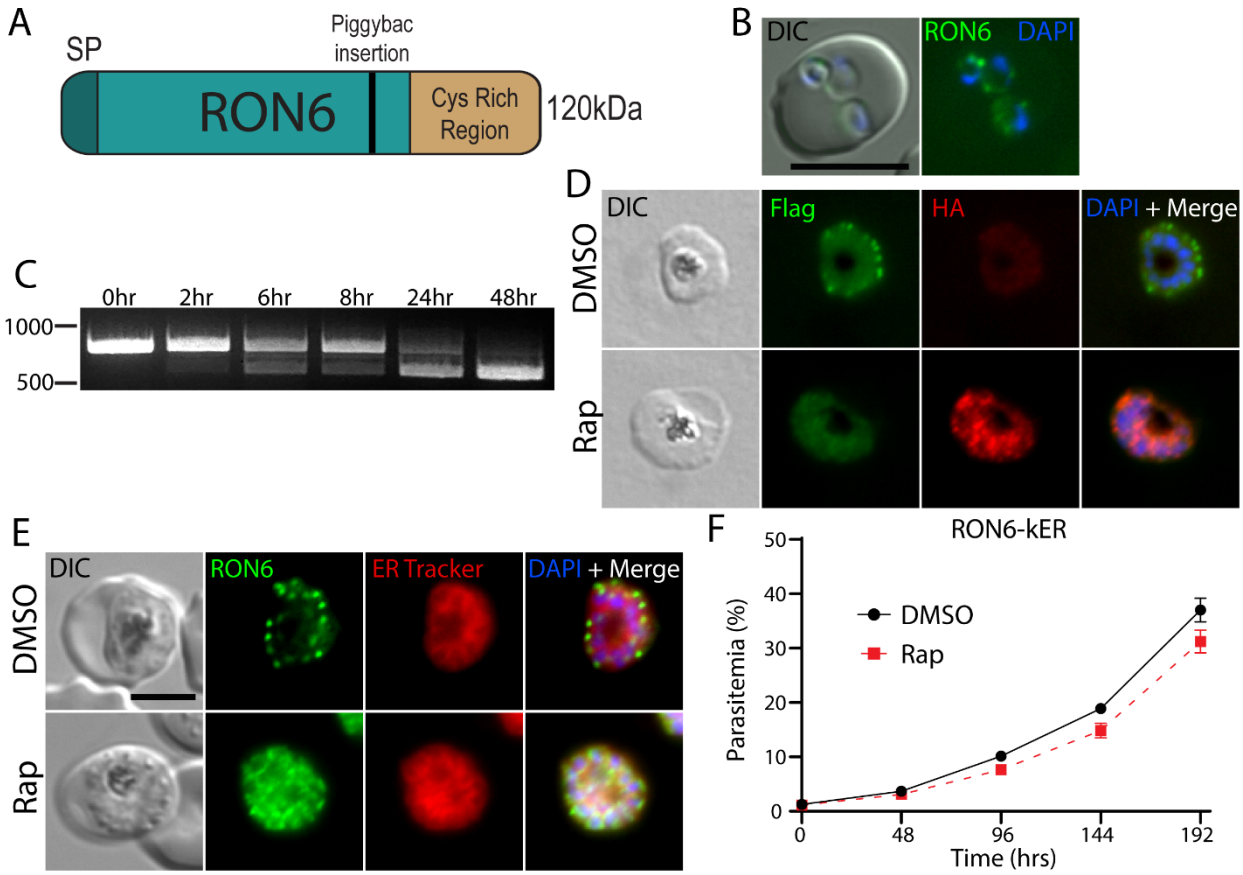

Figure S2. Inducible ER retention of rhoptry protein RON6. A) Schematic of rhoptry neck protein RON6. SP= signal peptide; black bar= site of piggyBac insertion in RON6 (21). B) Live microscopy of ring-stage RON6-kER parasites showing peripheral RON6-mNG signal, indicating RON6 localization to the PV after invasion. C) Time course of excision following rapamycin treatment detected by PCR using primers P7/8. D) Representative IFA of a RON6-kER schizont showing conversion from Flag to HA and retention of the HA-tagged species in the ER following rapamycin treatment. Synchronized parasites were treated with DMSO or rapamycin at 24 hpi and allowed to develop for 72 hrs to second cycle schizonts before fixation. At least 40 iRBCs were assessed across multiple fields in each condition; all rapamycin-treated parasites showed conversion to the HA-KDEL tag. E) Live microscopy of DMSO or rapamycin-treated RON6-kER parasites 48 hrs post-treatment, treated with 25 nM ML10 for 4hrs (BEI Resources) to prevent egress and arrest parasites as terminal schizonts. F) Representative growth of asynchronous RON6-kER parasites (n=3 biological replicates) treated with DMSO or rapamycin. Data are presented as means ± standard deviation from one biological replicate (n = 3 technical replicates). Scale bar, 5μm.

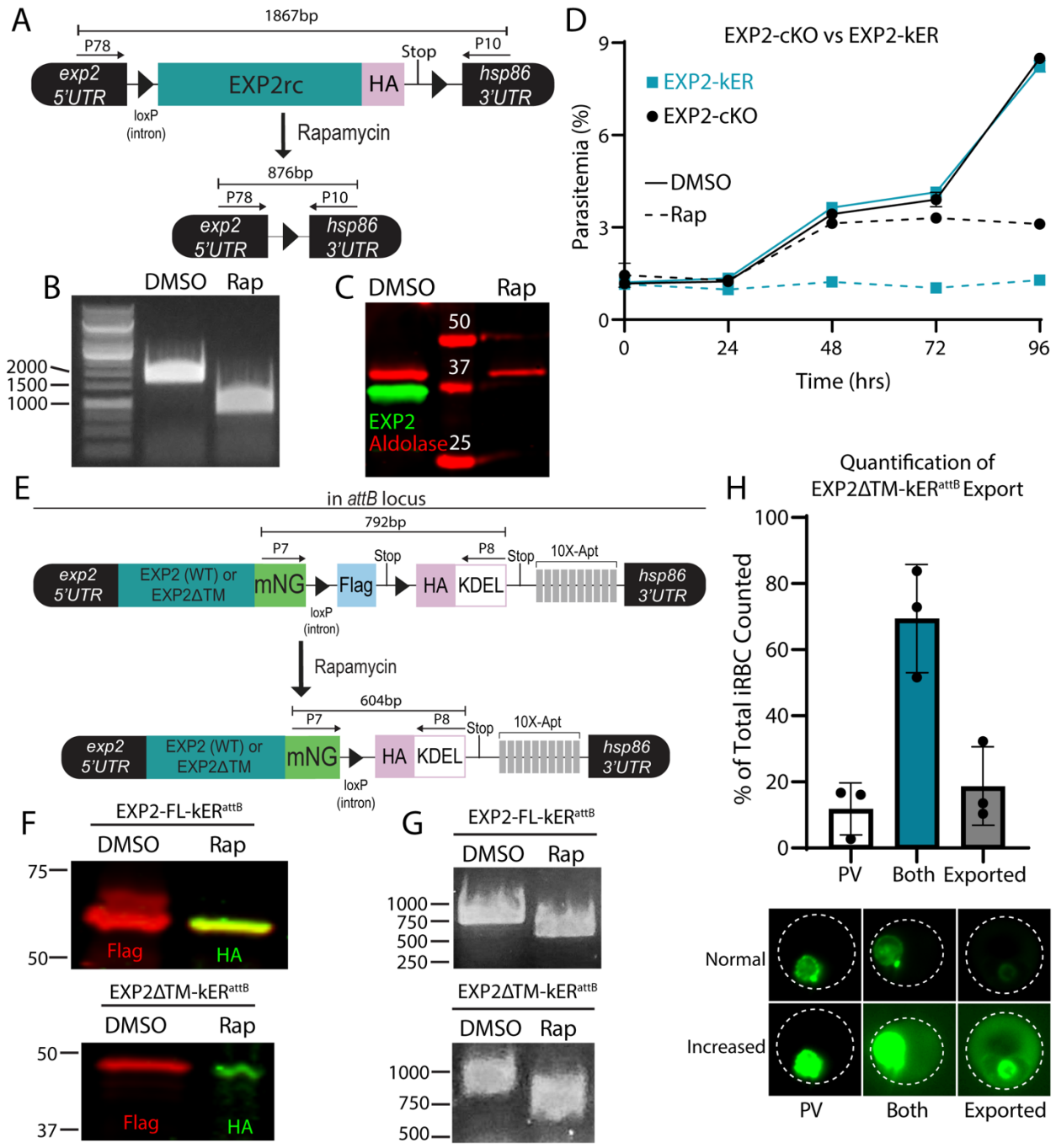

Figure S3. Supporting data for Figure 3. A) Schematic showing the modified, floxed endogenous *exp2* locus for DiCre-based conditional knockout in EXP2-cKO parasites. EXP2rc; recoded EXP2 sequence. B) PCR showing excision of the *exp2* gene in EXP2-cKO parasites 48 hours after rapamycin treatment using primers P78/P10. C) Western blot 48 hours after treatment of EXP2-cKO parasites with DMSO or rapamycin showing loss of EXP2 expression. Aldolase serves as a loading control. Molecular weights are predicted to be 40.1 kDa for aldolase and 33.8 kDa for EXP2-3xHA after signal peptide cleavage. D) Representative comparative growth assay of EXP2-kER and EXP2-cKO parasites (n=2 biological replicates). Synchronized, ring-stage parasites of each line were treated with DMSO or rapamycin at time 0. Data are presented as

means ± standard deviation from one biological replicate (n = 3 technical replicates). E) Schematic showing cassette for second copy expression of full-length EXP2 or a version lacking the amphipathic helix with kER fusion from the *attB* site of chromosome 6. F) Western blots from second copy EXP2-kER parasites maintained in media supplemented with 500 nM aTc. Samples were taken 24 hrs after DMSO or rapamycin treatment showing conversion from Flag to HA. Molecular weights after signal peptide cleavage are predicted to be 61.4 kDa for EXP2-FL-3xFLAG, 61.5 kDa for EXP2-FL-3xHA-KDEL, 54.4 kDa for EXP2ΔTM-3xFLAG and 54.5 kDa for EXP2ΔTM-3xHA-KDEL. G) PCR showing excision in EXP2-FL-kER^{attB} and EXP2ΔTM-kER^{attB} parasites 24 hrs after rapamycin treatment using primers P7/8. H) Quantification of EXP2ΔTM-mNG export in EXP2ΔTM-kER^{attB} parasites without rapamycin treatment (n=3 biological replicates). Infected RBCs (iRBCs) were scored as having strict PV retention without apparent export (PV), both exported and PV signal (both), or full export without apparent PV signal (full). Error bars indicate mean ± standard deviation. Representative images are shown below and displayed with normal or increased brightness and contrast to clearly show the fractions of mNG signal localized to the PV, digestive vacuole and RBC cytosol.

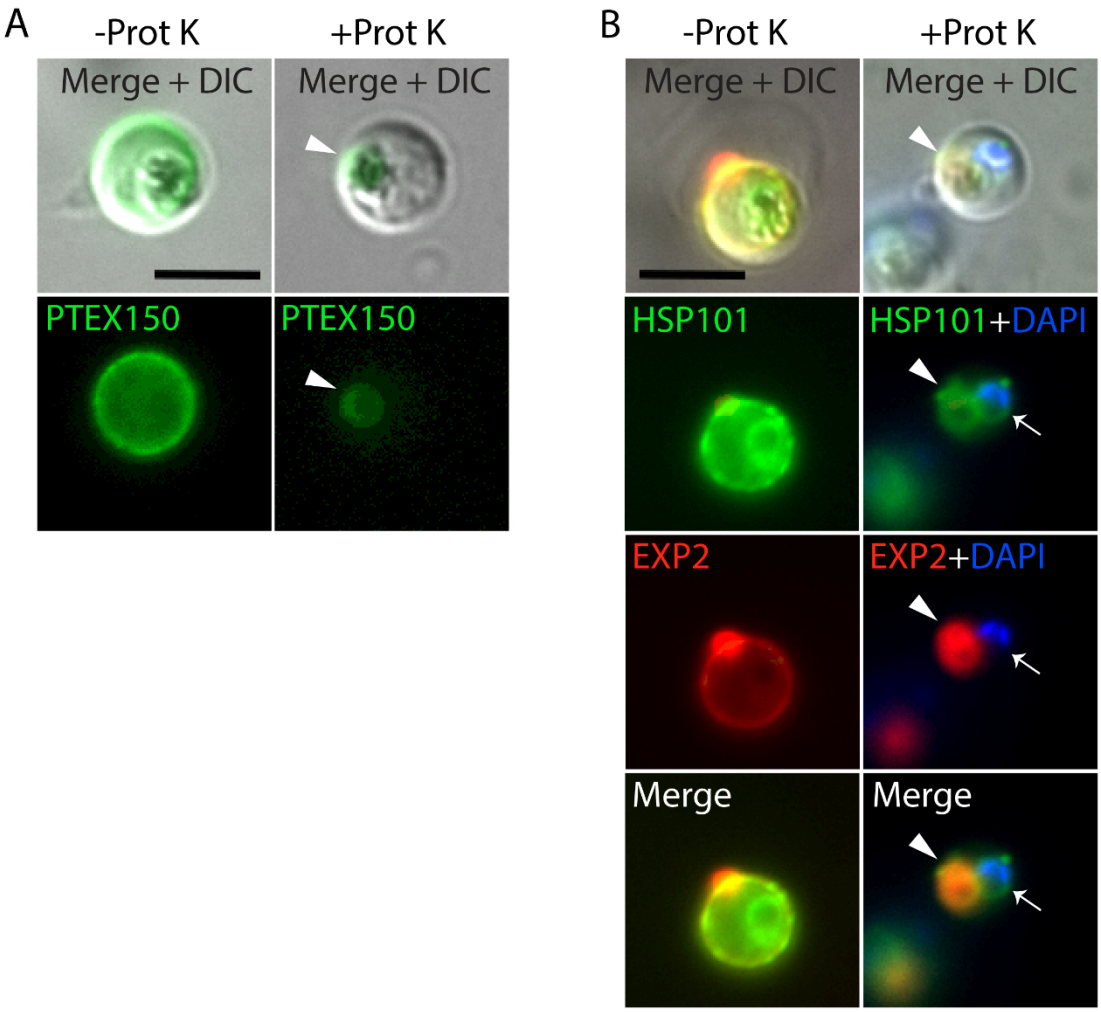

Figure S4. Protease protection assay on live cells verifies an internal, perinuclear pool of HSP101 but not EXP2 or PTEX150. Live microscopy of parasite lines with endogenous fluorescent protein fusions A) PTEX150-mNG-3xFLAG or B) EXP2-mRuby3 and HSP101-mNG treated with saponin +/- Proteinase K. Images are representative of n=2 biological replicates. Arrowhead: protease-protected fluorescence within the digestive vacuole from endocytosis typically seen for PV proteins; arrow: protease-protected perinuclear fluorescence unique to HSP101. Scale bar, 5μm.

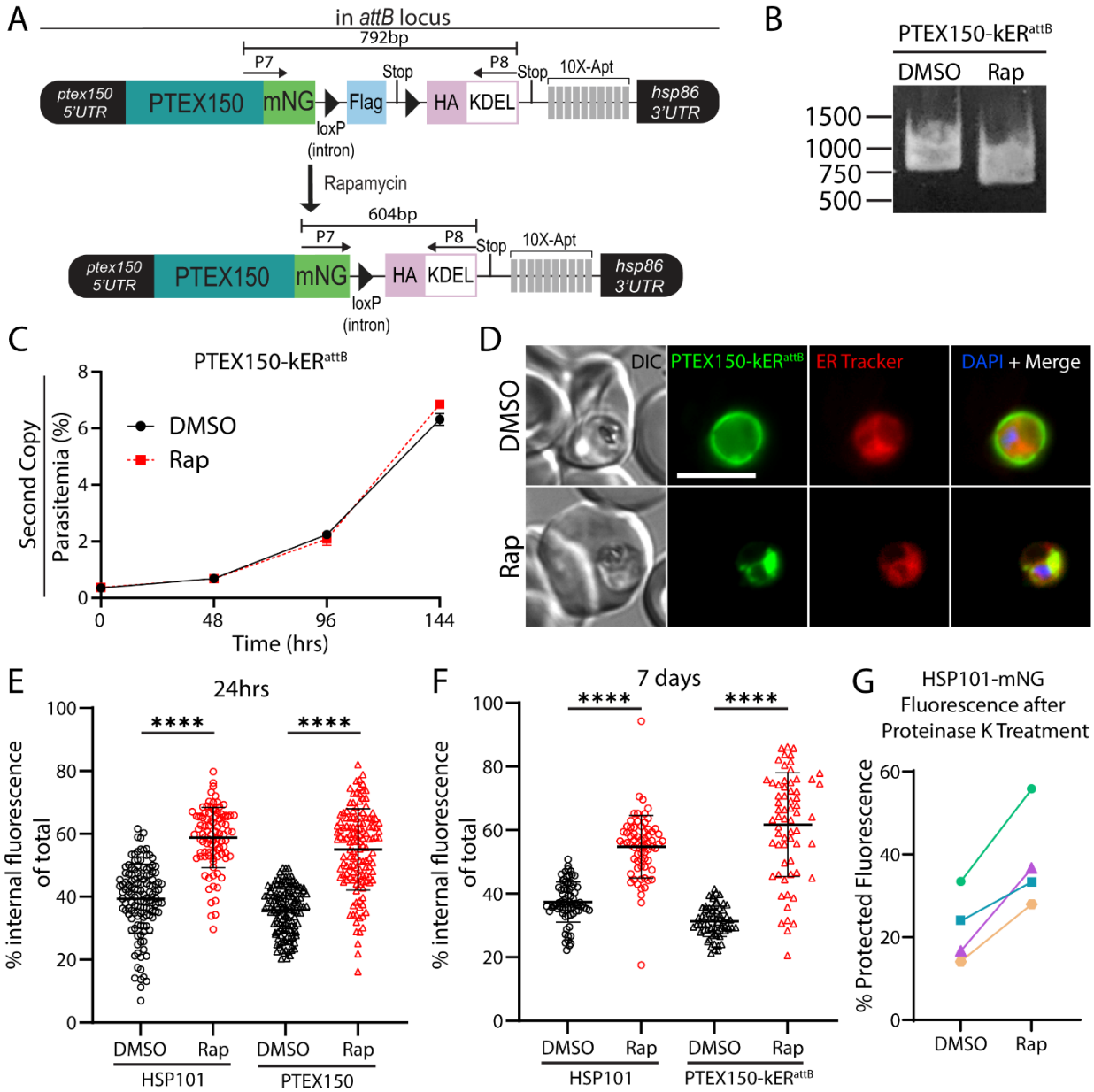

Figure S5. Supporting data for Figure 4. A) Schematic showing cassette for second copy expression of PTEX150-kER from the *attB* site of chromosome 6. B) PCR showing excision in PTEX150-kER^{attB} parasites 24hrs after rapamycin treatment using primers P7/8. C) Representative growth curves of asynchronous, second copy PTEX150-kER parasites (n=3 biological replicates) treated with DMSO or rapamycin. Data are presented as means ± standard deviation from one biological replicate (n = 3 technical replicates). D) Live microscopy of second copy PTEX150-kER parasites 24 hrs post-treatment with DMSO or rapamycin. PTEX150-kER^{attB} cultures were maintained in media supplemented with 500 nM aTc. E) Quantification of percent internal mNG fluorescence in live microcopy images of endogenously tagged HSP101- and PTEX150-kER parasites 24 hrs post-treatment with DMSO or rapamycin. Data are pooled from 3 (HSP101) or 2 (PTEX150) independent experiments and bars indicate mean ± standard deviation (****, P<0.0001; unpaired t test). F) Quantification of percent internal mNG fluorescence in live microcopy images of endogenously tagged HSP101-kER and second copy PTEX150-kER parasites lines 168 hrs post-treatment with DMSO or rapamycin. Data are pooled from 2

independent experiments and bars indicate mean ± standard deviation (****, P<0.0001; unpaired t test). G) Mean fluorescence intensity quantification from proteinase K treated parasites. The amount of protected fluorescent signal (percent of total before protease treatment) was determined by dividing the MFI after proteinase K treatment by the MFI before proteinase K treatment. Colors represent different replicates.

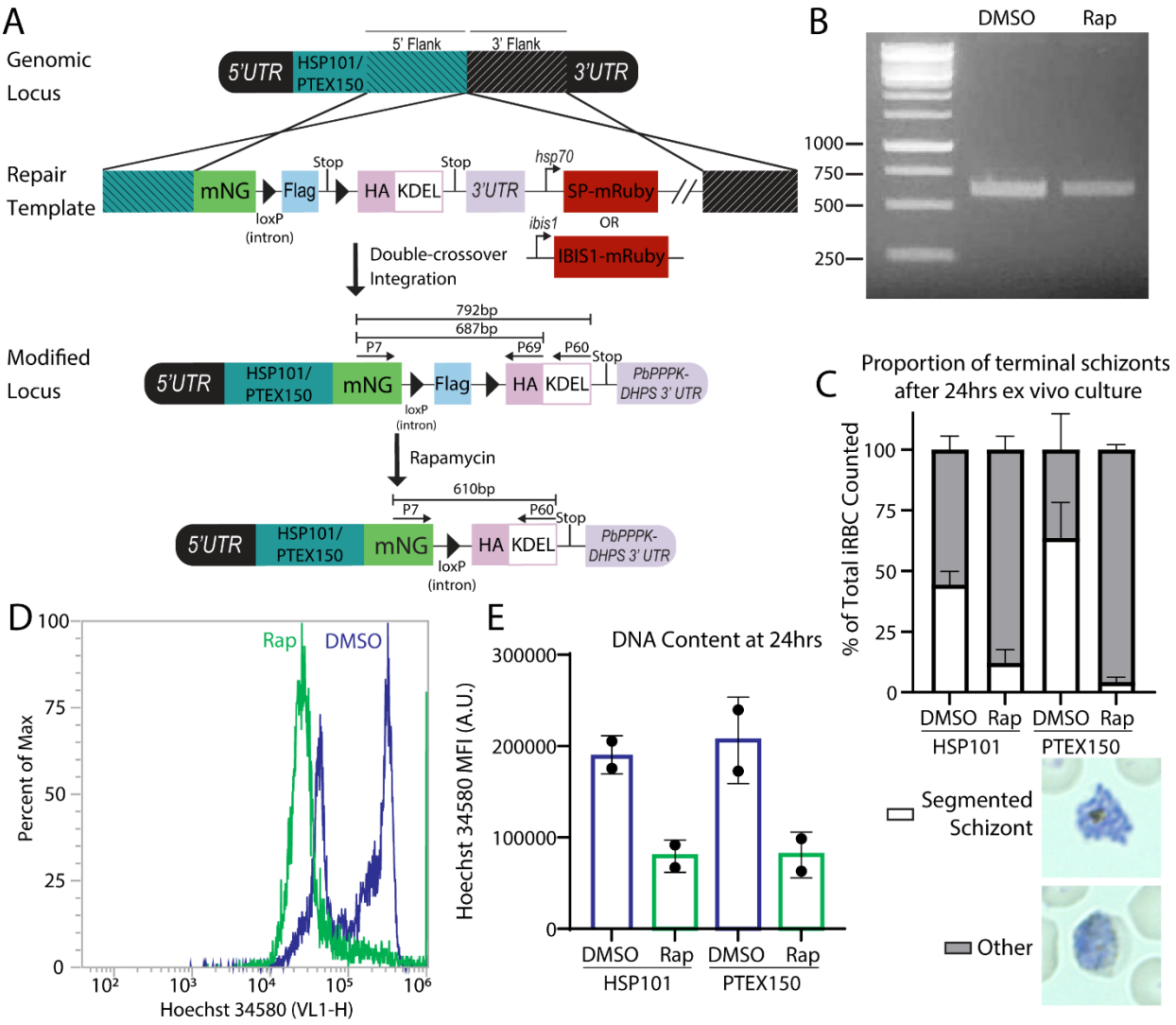

Figure S6. Supporting data for Figure 5. A) Schematic showing strategy for appending kER to the endogenous C-terminus of *P. berghei hsp101* and *ptex150*. Tagging plasmids include a downstream cassette for expression of mRuby fused to the EXP2 signal peptide for default secretion into the PV or IBIS1-mRuby, which is exported into the RBC. B) PCR using P7/P69 to evaluate excision of the kER cassette in parasites taken from mice injected with DMSO-treated PbHSP101-kER (collected on day 8) or rapamycin-treated PbHSP101-kER (taken from the single mouse where parasites appeared on day 10). The results indicate that the kER cassette was unexcised in the parasites observed in the mouse that became patent on day 10. C-E) Assessment of PbHSP101-kER and PbPTEX150-kER parasite development in *ex vivo* culture. Synchronous ring-stage parasites (≤3 hpi) treated with DMSO or rapamycin in the preceding cycle were allowed to develop *ex vivo* for 24 hrs. Parasite development was assessed by C) the proportion of terminal, segmented schizonts from Giemsa-stained smears (representative images shown below) and D,E) flow cytometry analysis of parasite DNA content. For flow cytometry analysis, 24 hr *ex vivo* cultures were stained with Hoechst 34580 and infected RBCs were gated using mNG fluorescence and Hoechst 34580 fluorescence (excited with a 405 nm laser) was collected from this population. D) Representative histogram of Hoechst 34580 fluorescence from one PbPTEX150-kER experiment. E) DNA content of rapamycin-treated parasites is substantially decreased compared to DMSO controls, in agreement with failure to normally complete schizogony and consistent with a major developmental defect in PbHSP101-KDEL and

PbPTEX150-KDEL during *ex vivo* culture. Points represent MFI from 5,000 iRBCs in each of two independent experiments. Error bars indicate standard deviation.

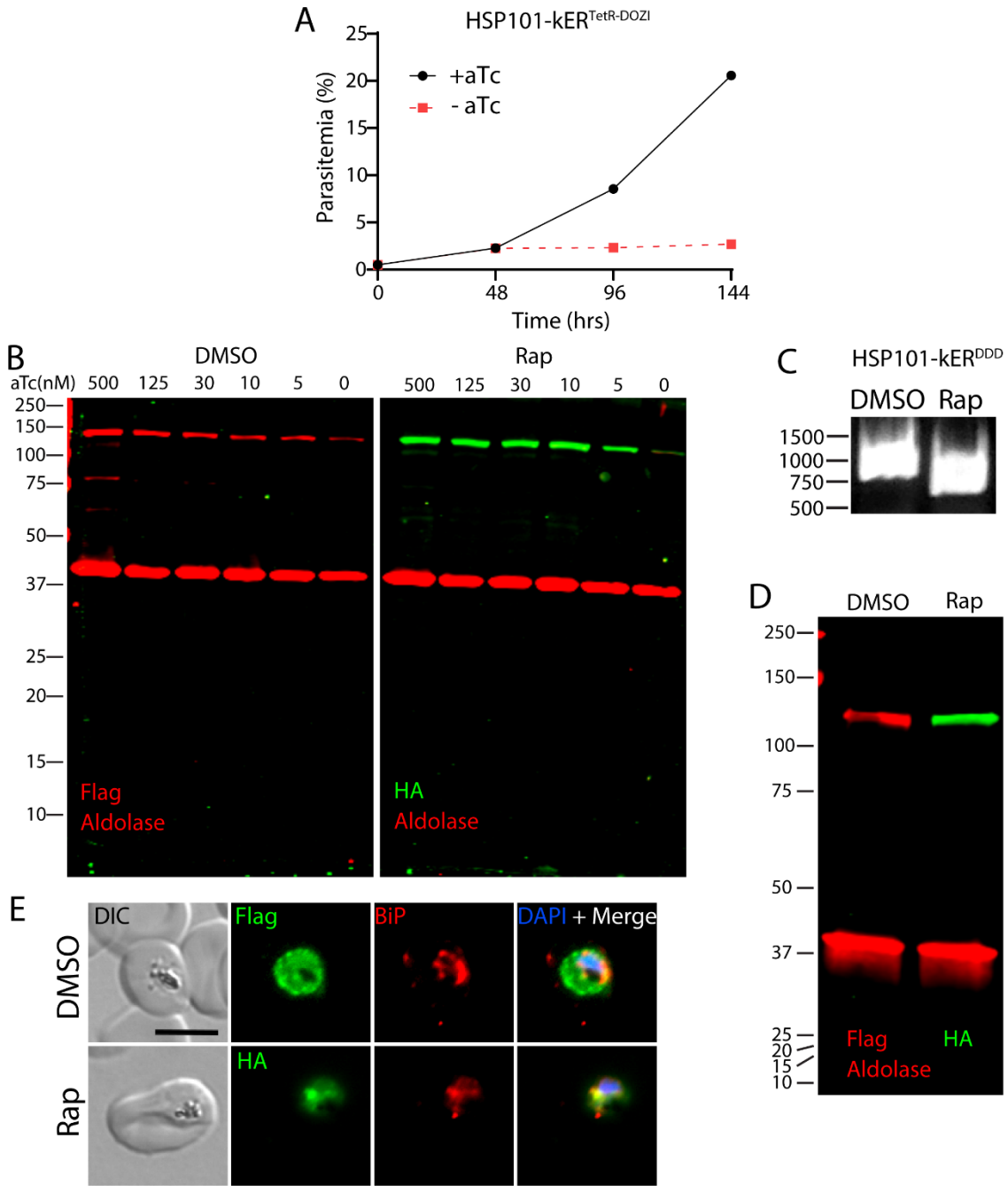

Figure S7. Supporting data for Figure 6. A) Representative growth curve (n = 3 biological replicates) of HSP101-kER$^{TetR\text{-}DOZI}$ parasites grown with or without aTc. Data are presented as means ± standard deviation of one biological replicate (n = 3 technical replicates). B) Western blot of HSP101-kER$^{TetR\text{-}DOZI}$ parasites grown 48 hrs at indicated aTc concentrations. Parasites grown in 500 nM aTc were treated with DMSO or rapamycin 24hrs before aTc was washed out and restored at indicated concentrations. Results are representative of 4 independent experiments. Molecular weights after signal peptide cleavage are predicted to be 130.5 kDa for HSP101-mNG-3xFLAG and 130.6 kDa for HSP101-mNG-3xHA-KDEL. C) PCR showing excision in HSP101-kER^{DDD} parasites 72hrs after rapamycin treatment using primers P70/8. D) Western blot of HSP101-kER^{DDD} parasites 2.5 cycles post-treatment with DMSO or rapamycin. Molecular weights after signal peptide cleavage are predicted to be 122.1 kDa for HSP101-DDD-3xFLAG, 122.2 kDa

for HSP101-DDD-3xHA-KDEL and 40.1 kDa for aldolase. E) IFA of HSP101-kER^{DDD} parasites 2.5 cycles post-treatment with DMSO or rapamycin. BiP serves as a marker for the ER.

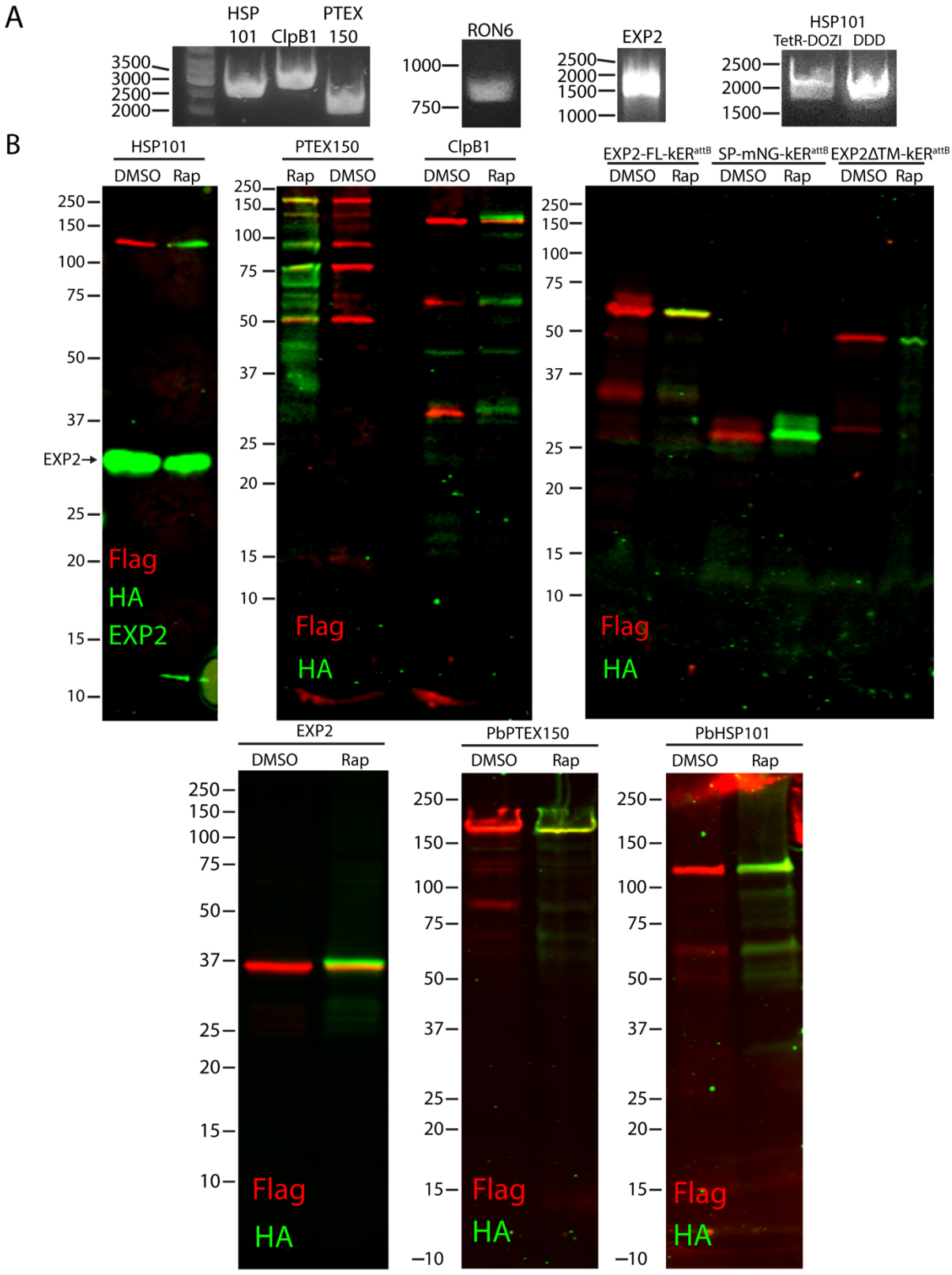

Figure S8. Integration PCRs and Uncropped Western blots A) Integration PCR for endogenous kER tags using primers P11/P12 (HSP101), P13/P12 (ClpB1), P14/P12 (PTEX150),

P15/P16 (RON6), P17/P8 (EXP2), P11/P12 (HSP101$^{TetR\text{-}DOZI}$), and P11/P71 (HSP101^{DDD}). B) Uncropped Western blots.

Table S1. Sequences of primers used in this study.

Name	Sequence
P1	ATTGCATACACATAAATATTTGTGTTGTAC
P2	CACATACGTATTGTGTTGAGCTTAATAG
P3	CTAATGTTAAGCCAACTGTAGTTGGG
P4	GATGCGATATGTAATTCCATTACTGC
P5	CCATTTACACATAAATGTCACACAAAAGAG
P6	GAAATAATTTCATATACACACATATAC
P7	GCTACACCTACGAGGGAAGC
P8	ATATATTAATAACTCGACGCGGCCGTTATAATTCGTCCTTGGCATAATCTGGAACATCGTAAGGATACG
P9	CAGATGACGACGATGATGATGACG
P10	GTATATTGGGGTGATGATAAAATGAAAG
P11	CGAAAACTTTTATGGTATTAATATAACAG
P12	GTAAGTCTTCTTCGACCTGCAC
P13	GATGAAATCCATACTGTTGTGGGAGC
P14	CGATGATGATGATGATTTGGATGAAG
P15	CTTGACATGAATGAAACCATACAAAAATTAAAATCCGATAGTG
P16	GACGATGTACATTTTGTACCTTCCCAATTTGTTACTATAAAAGTAAGACATC
P17	CACTCCTCCACTTCCCCTAGGTCCCGTTTTTTGTTTCTCGTGCAAGGGTAATACATACAACGTACCAATTGTGTC
P18	CAGATGACGACGATGATGATGACG
P19	ACCTAATAGAAATATATCACCTAGGATAACTTCGTATAGCATACATTATACGAAGTTATATGGTGAGCAAGGGCGAGGAG
P20	CCTTACGATGTTCCAGATTATGCCTGAATAACTTCGTATAGCATACATTATACGAAGTTATCTTAAGGTCGAGTTATATAATATAT
P21	CACTATAGAACTCGAGGGAGAAACAATCTTTTATATAAAATGTACAGAGTTTGAAAG
P22	GTGGAGATTTATTTAAAAATGAAAAAAAAGATGAAAATAAAGAACCTAGGGACTACAAGGACGACGACG
P23	AAAAAGATGAAAATAAAGAACCTAGGGTAAATAAAAAAAATAATATACAATAACTTCGTATAGCATACATTATACGAAG
P24	TCGTCGTCGTCCTTGTAGTCGCTAGCGTTGCCGTTATTCTGGCTAGCC
P25	AAGATGATGATGATAAATGAACTAGTATAACTTCGTATAGCATACATTATACGAAGTTATTATATATG
P26	CGTATCCTTACGATGTTCCAGATTATGCCAAGGACGAATTATAACGGCCGCGTCGAGTTATTAATATAT
P27	GATGAAAATAAAGAACCTAGGGGAAGTGGAGGAGTGAGCAAGGGCGAGGAGGATAAC
P28	ATATTATTTTTTTTATTTACCTTGTACAGCTCGTCCATGCCCATC
P29	TCCAATGGCCCCTTTCCGGGCGCGCCCCTTGTAAATTTTAATAGTTATAATATAATATTATTTACTTCTACATCCACTG
P30	CGCCCTTGCTCACTCCTCCGGATCCAACTACAGTGTTTGTATTTTTATATACGAAGAATAACAAAAAAAAGG
P31	GGATCCGGAGGAGTGAGCAAGGGCG
P32	CTCTGCCTTGCCAGACAGTGGGCCCTTATAATTCGTCCTTGGCATAATCTGGAACATCG
P33	CGCCCTTGCTCACTCCTCCGGATCCTTCTTTATTTTCATCTTTTTTTTCATTTTTAAATAAATCTCCACTGGC
P34	CATATTAAGTATATAATATTATTTTTAGAAAAATATTATGGTTTTAGAGCTAGAAATAGC
P35	CATATTAAGTATATAATATTTAATACATACAACGTACCAAGTTTTAGAGCTAGAAATAGC
P36	GTAACCTTGTGCATATCAGAAAATTGTTTGCACTCCTTAAGGATGAAGTTACAAGTGAAGATATTGTTAATATTGTAAGTATGTCTACC
P37	CACTCCTCCACTTCCCCTAGGACTTTTTGAAAAGTGCAACTTCTGATCTTTCAGAG
P38	AGCAATACATACATAATACCTTAAGGGATATAATTTACATGAATGTTTACAATTTTTGGTCGTC
P39	CACTCCTCCACTTCCCCTAGGTCCCGTTTTTTGTTTCTCGTGCAAGGGTAATACATACAACGTACCAATTGTGTC
P40	CACTATAGAACTCGAGCAAGTACATAAAGAAAATGCTTATCAATCATCGATTTA
P41	GGTAGACATACTTACAATATTAACAATATCTTCACTTGTAACTTCATCCTTAAGGAGTGCAAACAATTTTCTGATATGCACAAGGTTAC
P42	TTTAGGTGACACTATAGAACTCGAGCAATATGTATATATATATATATGCAAATGTATAAATCTACATATGCATATTGTC
P43	ATGTAAATTATATCCCTTAAGGTATTATGTATGTATTGCTTAATTTAATAATGATTATCCATTTTTTTAATTTTTG
P44	TTTAGGTGACACTATAGAACTCGAGGGTATAGAAAAAATATATAATATTTATATGCTTTTCTGCCAAATTTGC
P45	CACTCCTCCACTTCCCCTAGGGTTATCATCTTCTTCTTCGTCTAATTCTTCTTCATCATTAGAATCATCG
P46	GGTGACACTATAGAACTCGAGAATTACGCATATATATATATATATATATATATAACATGGGTTGTATAATTTAAAAG
P47	CACTCCTCCACTTCCCCTAGGGGTCTTAGATAAGTTTATAACTAAGTTTTTAGCTTTAC
P48	TCCAATGGCCCCTTTCCGGGCGCGCC
P49	CGCCCTTGCTCACTCCTCCGGATCCGGTCTTAGATAAGTTTATAACTAAGTTTTTAGCTTTACTATTATAATCAAC
P50	ATTAGCTAAGCATGCGGGCCCGTTGTTTTTCTCTTTGTGGTCAAAATAAGTAAAATTTTATAAATTC
P51	CTCGCCCTTGCTCACCTCGAGATTGTCGTCCTCTTCTTCGTCCAATTCTTCTTCATC
P52	CGACAATCTCGAGGTGAGCAAGGGCGAGGAGG
P53	CCTTACGATGTTCCAGATTATGCCAAGGACGAATTATAACTTAAGGTCGAGTTATATAAT
P54	TATAAACTTATCTAAGACCCCTAGGGGAAGTGGAGGAGTGAGCAAGG
P55	GGCATGGACGAGCTGTACAAGTGACGGCCGCGTCGAGTTATATAATATA
P56	AATATATAAGTAAGAAAAAACGCGTCCCGGGAAGCTTATCGATGG
P57	AATTACAATTACAATTATTTAAATATGAAGATTCGTTATTTTTTGTCTCTTTTTTTATTTTATGCGACCTATAAGCATACAACAGCTAGCCCATGGGGAAGTGGAGGAGTGTCTAAGGGC
P58	CTTGCACACCTTTTAGCTAGGATCCTTAGGCATAATCTGGAACATCGTAAGGATACG
P59	TACTTCTCGCGAGCGCGCCCGCGGGGAAGTGGAGGAGTGAGCAAGGG
P60	CGATGTTCCAGATTATGCCAAGGACGAATTATAATCTAGAACTTATTGAAGAAAAAAAT
P61	GCATTAGGATTAGATATTTGCGGTTTGGTACAACATATTAGCTTAAGCTCATAATGCTATGAATGTAGATTCACTTTATCAAGAGC
P62	TGCTCACTCCTCCACTTCCCCTAGGTGACAATGAAAGATTAATAACAATGTTGTTGATTGTTTTGTTGTAATCAACATATACATCCATATCATC
P63	GTAGCATGGTGTGATTTATATCTCCTCATTTGTTGAACCTTAAGCAATTAATTCAAGACAATATACTCGATTATAGTGATTTAACAG
P64	TGCTCACTCCTCCACTTCCCCTAGGTTCATCTTCATCTTCATCTTCTGGGTAATCTTCTAATAATTCATTATCACTGTTGCC
P65	TACTTCTCGCGAGCGCGCCCGCGGCAAACACGATATGTTGCATGTATATATATTTATCTTTTATTATTTGCTTATCG
P66	GCTCTTGATAAAGTGAATCTACATTCATAGCATTATGAGCTTAAGCTAATATGTTGTACCAAACCGCAAATATCTAATCCTAATGC
P67	TACTTCTCGCGAGCGCGCCCGCGGGAATATTGCTTTCCCTATATATAATTATAAAATTGAAAAAAGAGAAGCTGAC
P68	CTGTTAAATCACTATAATCGAGTATATTGTCTTGAATTAATTGCTTAAGGTTCAACAAATGAGGAGATATAAATCACACCATGCTAC
P69	CCGGGACGTCGTACGGG
P70	TATAAACTTATCTAAGACCCCTAGGGGAAGTGGAGGAATCAGTCTGATTGCGGCGTTAGCGGTAGATC
P71	ATTTTTTTTATTTACTGTACATCGCCGCTCCAGAATCTCAAAGCAATAGCTGTGAGAGTTC
P72	TTTGTTGTGCTAAGCACCAGCTGGAATGTCGATGTTTTATAAGGGCATAAAAATGTAG
P73	TTTCCTTGATCAGCTCTTCGCCCTTAGACACTCCTCCACTTCCGGCGCCCATAGGTTTTGCTCTACAAAATATGTTAGATTTATTCAAATTTTTTCTTTC
P74	GGAAGTGGAGGAGTGTCTAAGGGC
P75	CTTGCACACCTTTTAGCTAGGATCCTTACTTGTACAGCTCGTCCATGCCAC
P76	AAAGATGAAAATAAAGAACCTAGGATAACTTCGTATAGCATACATTATACGAAGTTATGCTAGCATGAAAGTAAGCTATATCTTTTCCTTTTTTCTGTTG
P77	TAATAACTCGACGCGGCCGATAACTTCGTATAATGTATGCTATACGAAGTTATACTAGTTCAAGCGTAATCAGGAACGTCGTAG
P78	ATGTACTCTCCTTATGAGGCGCGCCGAGCACGTTTTTTGTTATTTAATTATAATTTCTACTTTTTAGTTATTATTATTATAAATATTTG
P79	CAATACTATATCATTTTATTTTCTTTTCTCTTATATTATATTATTTAATTTTATCTTATTTTTTTTTTTTTTAAACCTAGGATAACTTCGTATAGCATAC

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