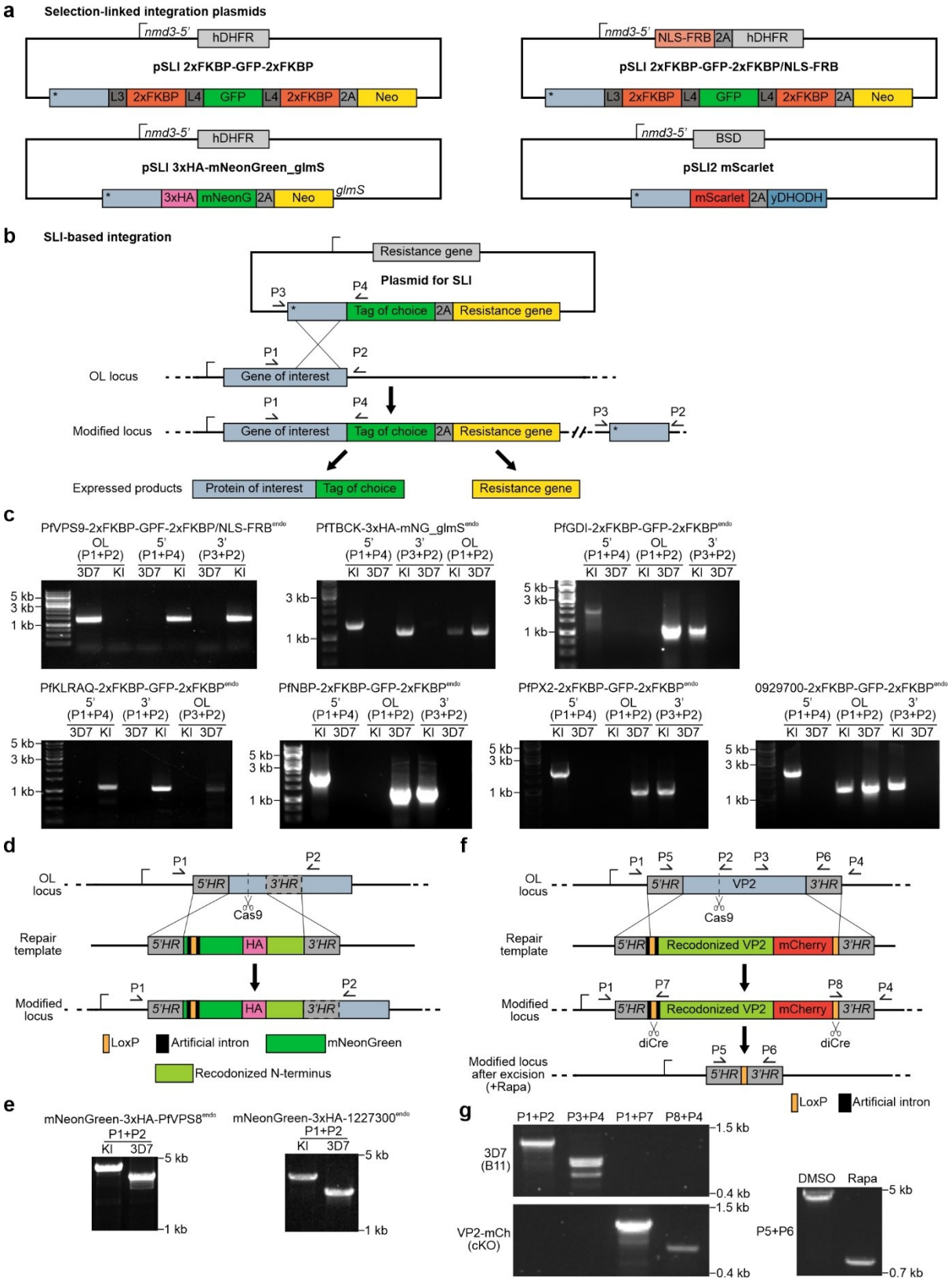
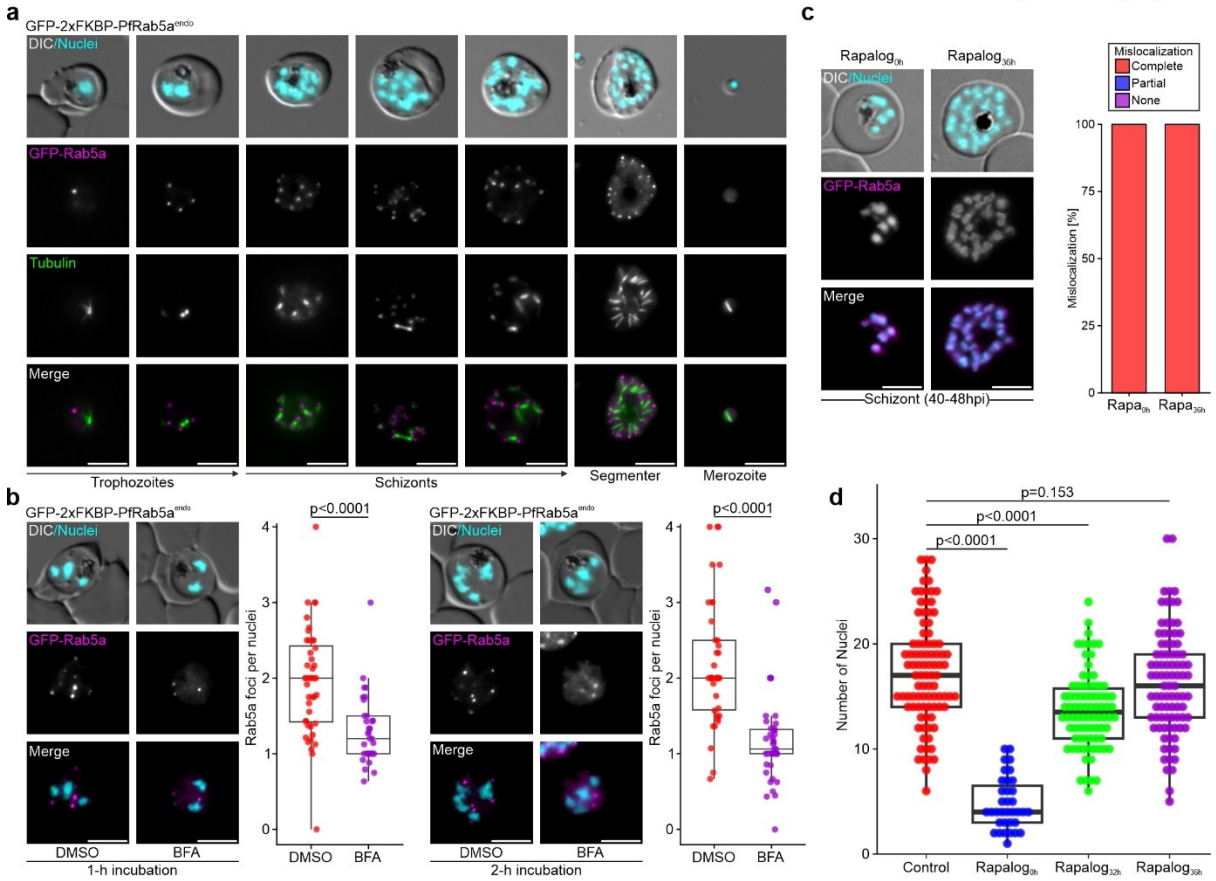
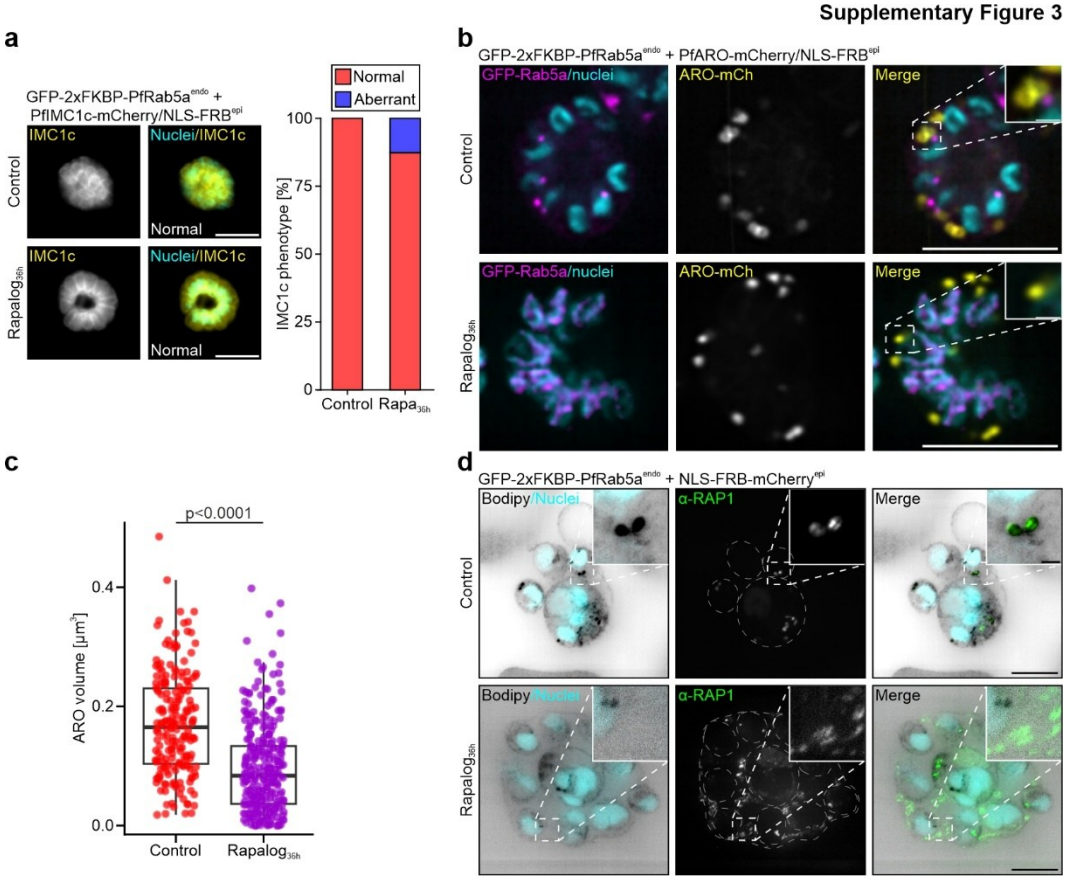


Supplementary Figure 1. Transgenic *P. falciparum* line generation. a, Schematic of selection-linked integration (SLI) constructs used to modify the parasite genome to express the C-terminally-tagged fusion proteins. HR, homology region; L3 and L4, linkers; 2A, T2A skip peptide; FKBP, FK506-binding protein; 3xHA, triple-HA tag; hDHFR, dihydrofolate reductase; BSD-R, Blasticidin S resistance; Neo-R, neomycin (G148) resistance; yDHODH,

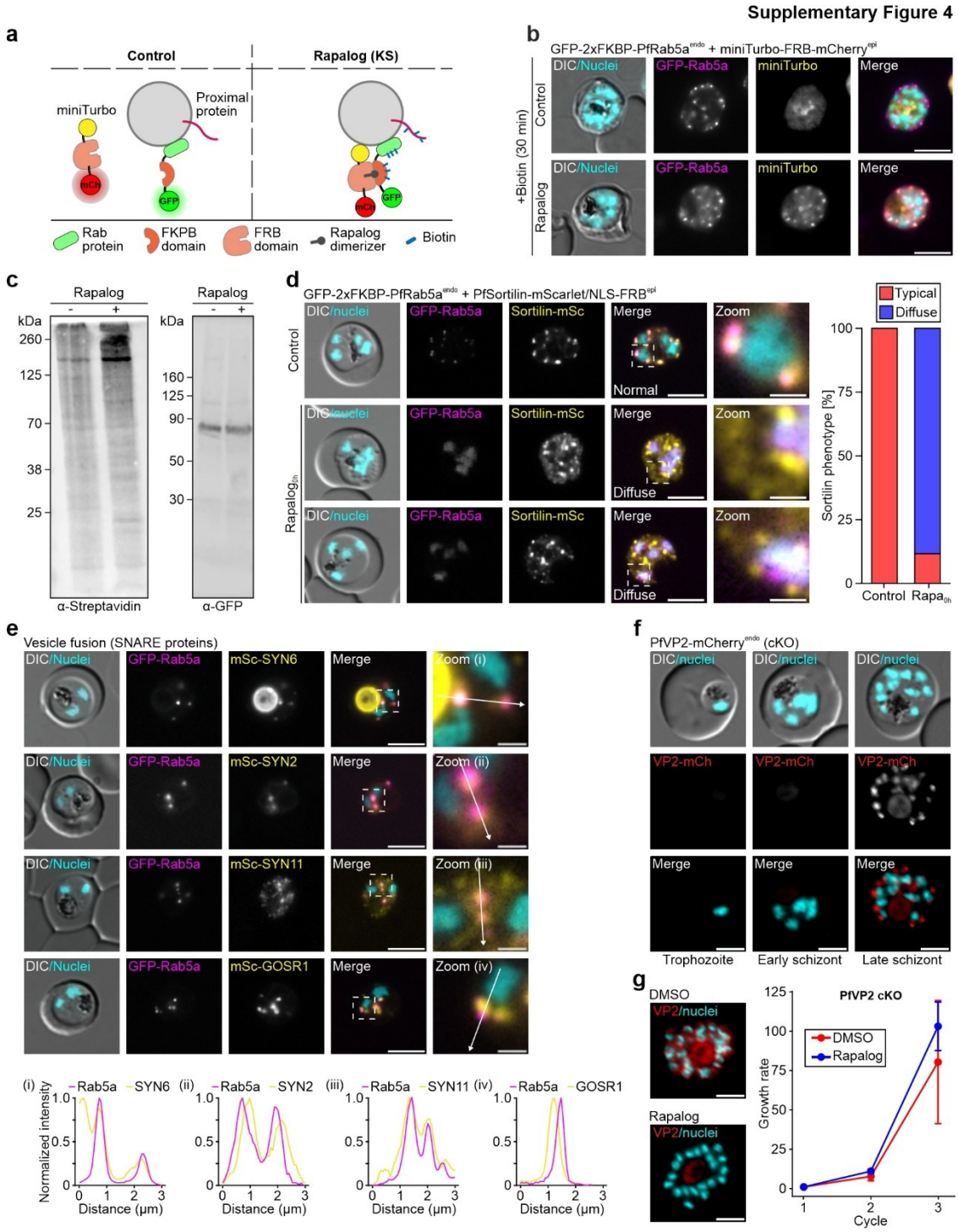
yeast dihydroorotate dehydrogenase; asterisk (*), stop codon; glmS, glucosamine-6-phosphate riboswitch ribozyme. **b,** Schematic of SLI strategy for generating stable transgenic parasites. Arrows, forward (P1, P3) and reverse (P2, P4) primers; OL, original locus. **c,** Agarose gels with PCR products showing correct integration of the plasmids from genomic DNA (gDNA) of 3D7 parasites and knock-in (KI) cell lines obtained after SLI. PCR products show 5' integration using primers P1 and P4, 3' integration using primers P3 and P2, and OL using primers P1 and P2. Primer sequences are shown in Supplementary Data 2. **d,** CRISPR-Cas9-based strategy for inserting a mNeonGreen-3xHA tag in the N-terminus of PfVPS8 and PF3D7_1227300. Arrows, forward (P1) and reverse (P2) primers. HA, triple-HA tag (3xHA); LoxP, LoxP sites. **e,** Agarose gel with PCR products showing correct insertion from 3D7 gDNA and KI lines after CRISPR-Cas9 editing. Primer and guide sequences are shown in Supplementary Data 2 and 3. **f,** CRISPR-Cas9 based strategy to generate PfVP2-mCherry conditional knockout (cKO) line and gene excision after DiCre recombinase-mediated gene excision. Arrows, forward (P1, P3, P5, P8) and reverse (P2, P4, P6 and P7) primers. **g,** Agarose gel with PCR products showing correct insertion (left) from 3D7 (B11) gDNA and KI lines after CRISPR-Cas9 editing endogenously expressing DiCre. Agarose gel on the right (P5+P6) shows altered locus under control conditions (dimethyl sulfoxide, DMSO) and after rapalog-induced DiCre activation (Rapa), confirming successful PfVP2 gene excision. PCR products show 5' integration using primers P1 and P7, 3' integration using primers P8 and P4, and OL using primers P1 and P2 for 5' and P3 and P4 for 3'. Genomic sequences of the SLI plasmids, and CRISPR and repair plasmids are provided in Supplementary Data 4 and 5.



Supplementary Figure 2. Additional data for characterization of PfRab5a compartment and PfRab5a KS efficiency. a, Live-cell fluorescence microscopy of PfRab5a^{endo} parasites incubated with TubulinTracker^{TM} DeepRed throughout the intraerythrocytic development cycle (IDC). **b,** Distribution of PfRab5a signal in PfRab5a^{endo} parasites after 1-hour or 2-hour incubation with 5 µg/mL brefeldin A (BFA). Quantification shows the number of PfRab5a foci per nuclei in BFA-treated and control (DMSO) parasites. **c,** Efficiency of KS in PfRab5a^{endo}+NLS-FRB-mCherry parasites. KS induction was performed at 0-4 hpi (Rapa$_{0h}$) or at 36-40 hpi (Rapa$_{36h}$) and imaged at 40-48 hpi. Results from n=30 parasites per condition from at least three independent experiments. **d,** Number of nuclei in 44-48 hpi schizonts upon KS induction at different timepoints: 0-4 hpi (Rapalog$_{0h}$), 32-36 hpi (Rapalog$_{32h}$) and 36-40 hpi (Rapalog$_{36h}$). DIC, Differential interference contrast; Nuclei, Hoechst 33342; Merge, overlay of magenta and green or magenta and cyan channels. Scale bars, 5 µm. Mean with SD; p-value, unpaired t-test.



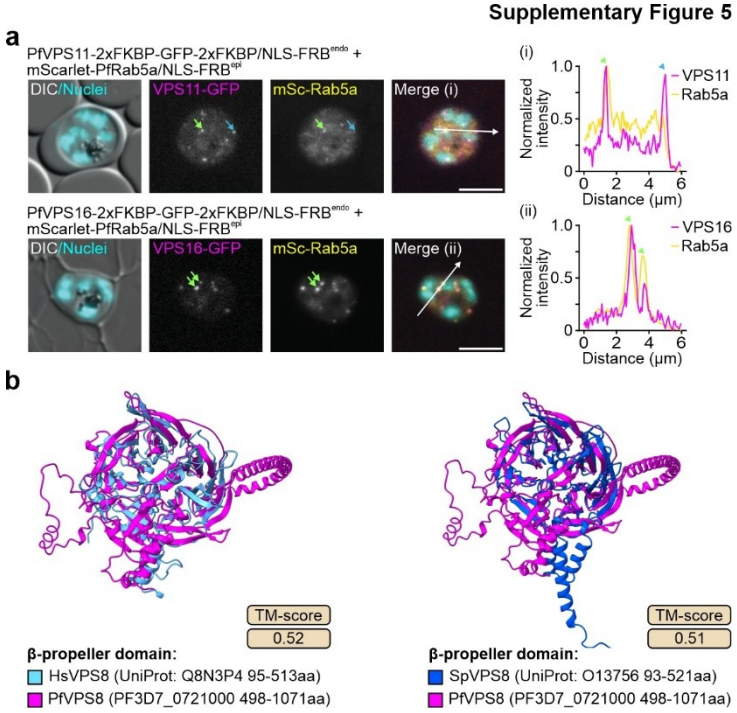
Supplementary Figure 3. PfRab5a loss alters localization of rhoptry bulb proteins. a, Live-cell fluorescence microscopy of KS and control PfRab5a^{endo} schizonts episomally expressing PfIMC1c-mCh. Graph on the right shows the PfIMC1c phenotype, n=20 cells per condition from two independent experiments. **b,** SoRa spinning disk confocal microscopy of KS (Rapalog$_{36h}$) and control PfRab5a^{endo} schizonts episomally co-expressing PfARO-mCh, and imaged at 48-52 hpi. Compound 2 (C2) was added to prevent egress. Boxed areas were enlarged to display ARO signal. **c,** Quantification of ARO-mCh volume from (**b**). Data n=20 schizonts per condition from two independent experiments. Mean with SD; p-value, unpaired t-test. **d,** Ultra-structure Expansion Microscopy (U-ExM) of control and KS (Rapalog$_{36h}$) Rab5a^{endo} parasites incubated with C2 until 44-48 hpi. U-ExM parasites were stained with Bodipy-TR (membranes) and Hoechst 33342 (nuclei) and specific antibodies for rhoptry-associated protein 1 (α-PfRAP1). Boxed areas were enlarged. SoRa, Super-resolution by optical reassignment. Nuclei, Hoechst 33342; Merge, overlay of magenta, yellow and cyan channels. Scale bars, 1 µm in (**b**, **d**, enlargements) and 5 µm in (**a**, **b**, **d**, whole cell).



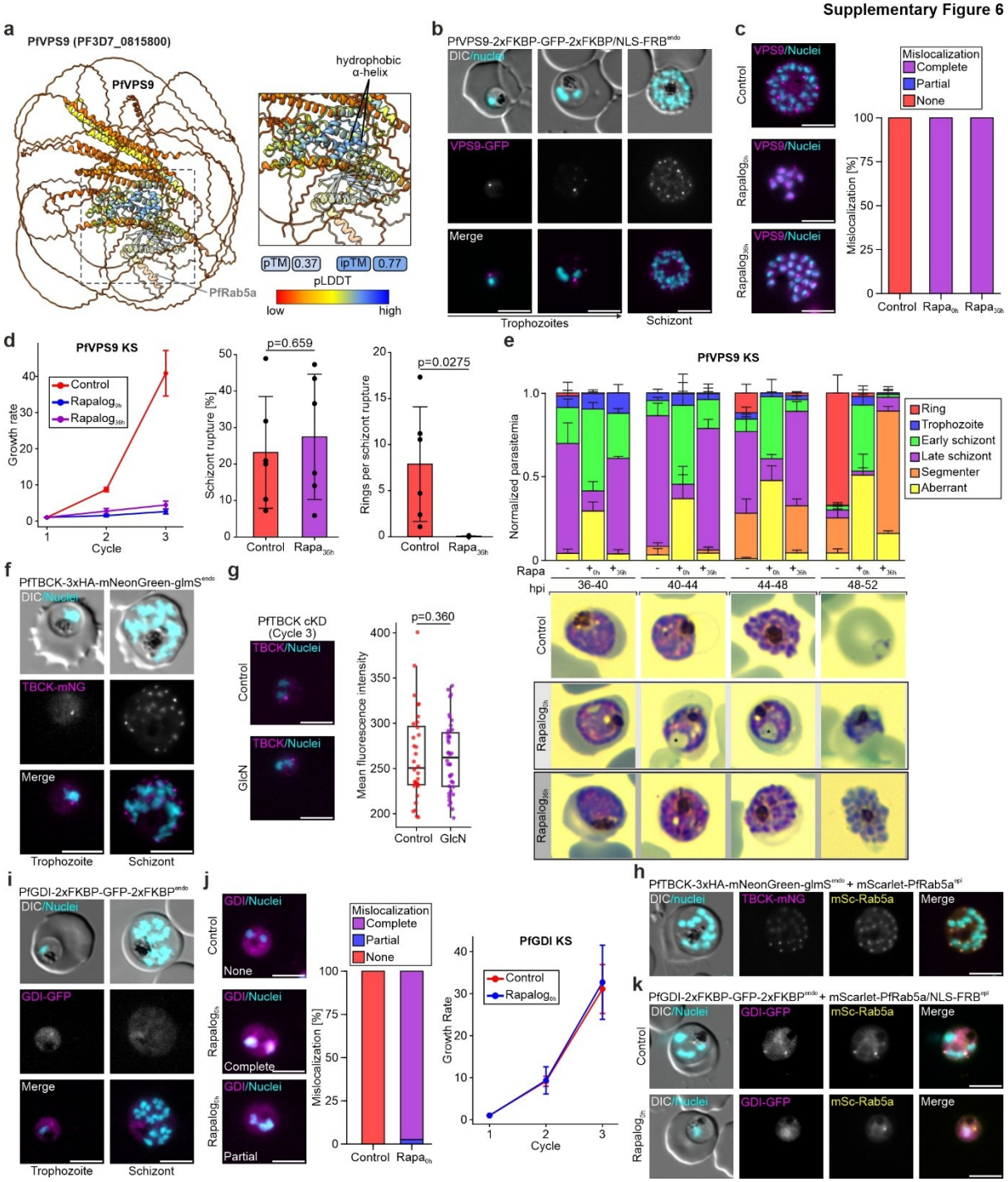
Supplementary Figure 4. Validation of candidates identified in the PfRab5a proxiome. **a,** Schematic of dimerization-induced quantitative proximity biotinylation-dependent identification (DiQ-BioID)[1]. The biotin ligase miniTurbo was C-terminally tagged with FRB-mCherry (miniTurbo-FRB-mCherry) and expressed in PfRab5a^{endo} parasites. In absence of rapalog (control), the biotin ligase is detected in the parasite cytosol. Upon addition of rapalog, miniTurbo was recruited to PfRab5a foci through FKBP-FRB dimerization, enabling proximity-dependent biotinylation of PfRab5a proximal proteins. **b,** Live-cell fluorescence microscopy of PfRab5a^{endo} parasites co-expressing miniTurbo-FRB-mCherry in the presence of rapalog or not (control) for 50 minutes. **c,** Representative western blot of parasite lysates from DIQ-BioID samples incubated with α-Streptavidin and α-GFP to show protein biotinylation and fusion

protein (GFP-2xFKBP-Rab5a^{endo}, 81 kDa) after 30-minute Biotin pulse incubation, respectively. kDa: kilodaltons. **d,** Live-cell fluorescence microscopy of control and KS ($Rapalog_{0h}$) PfRab5a^{endo} parasites episomally expressing PfSortilin-mSc at 30-38 hpi. PfSortilin phenotype is shown on the right and is defined as “Typical” (trans-Golgi localization) and “Diffuse” (Golgi-like localization together with diffuse pattern). **e,** Live-cell fluorescence microscopy of PfRab5a^{endo} parasites episomally co-expressing mSc-PfSYN6, mSc-PfSYN2, mSc-PfSYN11 and mSc-PfGOSR1. SYN, syntaxin; GOSR1, Golgi SNAP receptor complex member 1. **f,** Live-cell fluorescence microscopy of PfVP2-mCh^{endo} cKO parasites at trophozoite and schizont stages. **g,** Live-cell fluorescence microscopy and growth curve of PfVP2-mCh control (DMSO) or cKO (Rapalog) parasites. Growth rate was normalized to starting parasitemia and measured over three cycles by flow cytometry. DIC, Differential interference contrast; Nuclei, Hoechst 33342; Merge, overlay of magenta, yellow and cyan or red and cyan channels. Scale bars, 1 µm in (**d**, **e**, enlargements) and 5 µm in (**b**, **d**-**g**, whole cell).

Supplementary Figure 5

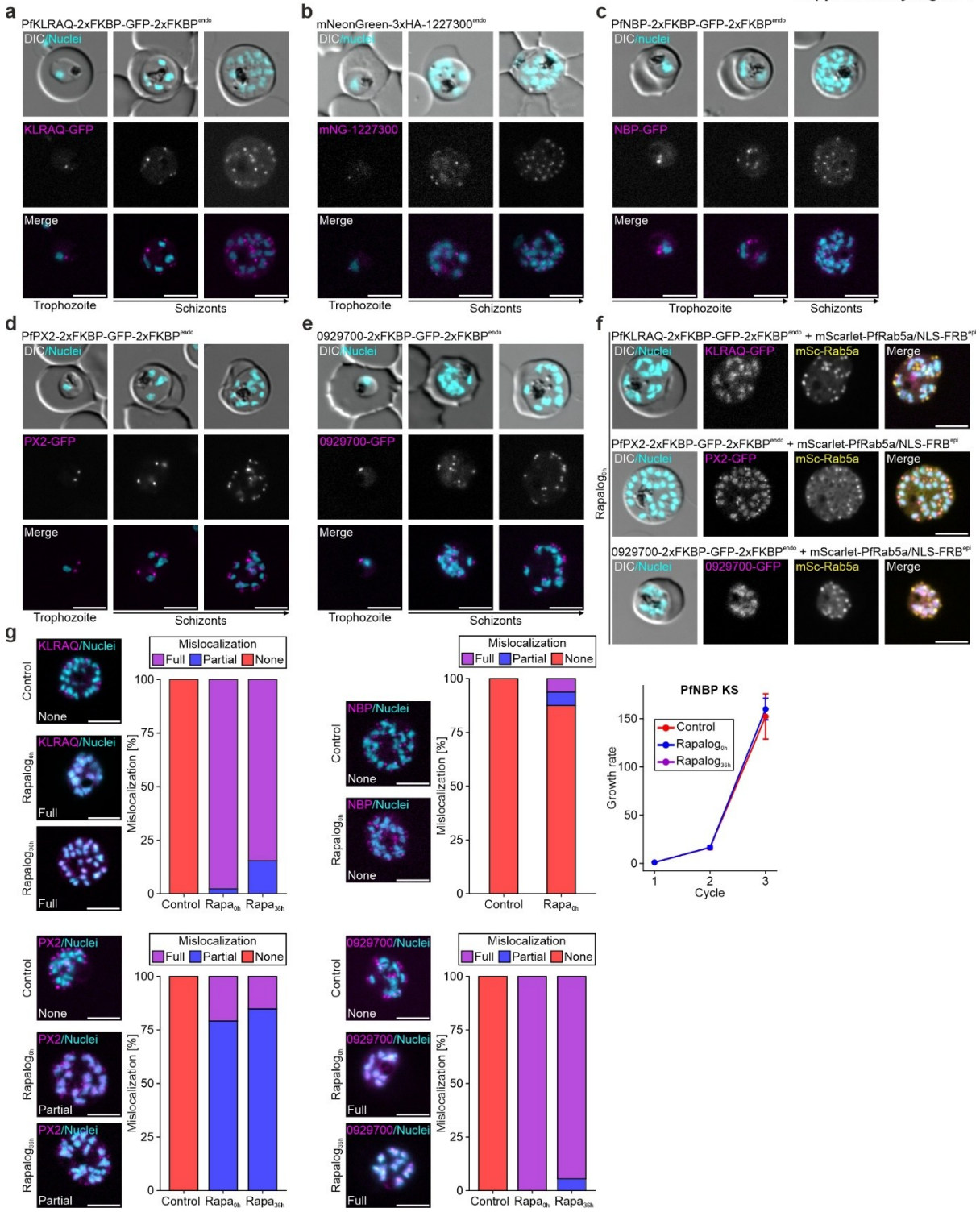


Supplementary Figure 5. Validation of HOPS/CORVET subunit candidates identified in the PfRab5a proxiome. a, Live-cell fluorescence microscopy of PfVPS11/NLS^{endo} and PfVPS16/NLS^{endo} schizonts episomally co-expressing mSc-PfRab5a. Normalized fluorescence intensity plots are shown on the right (i and ii). Green and blue arrowheads indicate colocalization or not with mSc-PfRab5a, respectively. DIC, Differential interference contrast; Nuclei, Hoechst 33342; Merge, overlay of magenta, yellow and cyan channels. Scale bars, 5 μm. **b,** Structure alignment of the predicted β-propeller domain of PfVPS8 (PF3D7_0721000, 498-1071aa, magenta) with the N-terminal VPS8 β-propeller domains of HsVPS8 (UniProt: Q8N3P4 95-513 aa, light blue) and SpVPS8 (UniProt: O13756 93-521 aa, dark blue). Structures were modelled using AlphaFold3[2]. Template modelling scores (TM-score) were calculated with FoldSeek; TM-scores > 0.5 indicate high structural similarity between compared structures[3]. Hs, *Homo sapiens*; Sp, *Schizosaccharomyces pombe*.



Supplementary Figure 6. Characterization of PfRab5a regulators. a, PfVPS9-PfRab5a interaction predicted by AlphaFold3[2]. Modelling shows all residues colored by predicted local distance difference test (pLDDT). Boxed area was enlarged, displaying the predicted interaction of PfRab5a with two hydrophobic α-helixes of VPS9 domain. **b,** Live-cell fluorescence microscopy of PfVPS9/NLS^{endo} parasites. **c,** Efficiency of KS in PfVPS9/NLS^{endo} parasites after addition of rapalog at 0-4 hpi ($Rapa_{0h}$) or at 36-40 hpi ($Rapa_{36h}$). Parasites were imaged at 40-48 hpi. Efficiency was defined as full, partial or no (none) mislocalization (n=15 parasites per condition from three independent experiments). **d,** Growth curve (left), and egress (middle) and invasion (right) assays in control and KS (Rapalog) VPS9/NLS^{endo} parasites. Growth rate was normalized to starting parasitemia and measured over three cycles by flow cytometry. Parasite egress and invasion capability measured by schizont rupture rate

(middle) and rings generated per schizont ruptured (right), respectively. Parasitemia was measured by flow cytometry. **e,** Blood-stage quantification of Giemsa smears in control and KS (Rapalog) VPS9/NLS^{endo} parasites. Bottom panel shows representative images of Giemsa smears. Asterisk (*) indicates abnormal large vacuolar structure. **f,** Live-cell fluorescence microscopy of TBCK^{endo} parasites. **g,** Efficiency of cKD in TBCK-glmS^{endo} parasites grown in control conditions (0 mM GlcN) or after cKD induction at 0-4 hpi (2.5 mM GlcN). Mean signal intensity of GFP signal from fluorescence microscopy parasites grown for three cycles under control conditions or with 2.5 mM GlcN. **h,** Live-cell fluorescence microscopy of TBCK-glmS^{endo} schizonts episomally expressing mSc-PfRab5a. **i,** Live-cell fluorescence microscopy of GDI^{endo} parasites. **j,** Efficiency of KS in PfGDI^{endo} parasites after induction at 0-4 hpi (Rapalog$_{0h}$) and analyzed at 32-36 hpi. Results from n=15 parasites per condition from two independent experiments. Representative microscopy images showing full and partial GDI mislocalization. Growth curve of PfGDI^{endo} parasites grown in control conditions or after KS induction at 0-4 hpi (Rapalog$_{0h}$). Growth rate normalized to starting parasitemia and measured over three cycles by flow cytometry. **k,** Live-cell fluorescence microscopy of control and KS (Rapalog$_{0h}$) PfGDI^{endo} schizonts episomally co-expressing mSc-PfRab5a. DIC, Differential interference contrast; Nuclei, Hoechst 33342; Merge, overlay of magenta and cyan or magenta, yellow and cyan channels. Scale bars, 5 µm. Mean with SD; p-value, unpaired t-test.



Supplementary Figure 7. Subcellular localization and KS efficiency of candidate Rab5a effectors. a-e, Live-cell fluorescence microscopy of PfKLRAQ^{endo} (a), 1227300^{endo} (b), PfNBP^{endo} (c), PfPX2^{endo} (d) and 0929700^{endo} (e) in trophozoite and schizont stages. **f,** Live-cell fluorescence microscopy of PfKLRAQ^{endo}-, PfPX2^{endo}- and 0929700^{endo}-KS (Rapalog$_{0h}$) schizonts episomally co-expressing mSc-PfRab5a. **g,** Efficiency of knock sideways (KS) in PfKLRAQ^{endo}, PfNBP^{endo}, PfPX2^{endo} and 0929700^{endo} parasites episomally expressing the nuclear mislocalizer. KS induction was performed at 0-4 hpi (Rapa$_{0h}$) or at 36-40 hpi (Rapa$_{36h}$) and analyzed at 40-48 hpi. Results from n=15 parasites per condition from at least two

independent experiments. Representative Live-cell fluorescence microscopy images showing full, partial or no (none) mislocalization for protein candidates. Growth curve of control and KS ($Rapalog_{0h}$) $PfNBP^{endo}$ parasites. Growth rate was normalized to starting parasitemia and measured over three cycles by flow cytometry. DIC, Differential interference contrast; Nuclei, Hoechst 33342; Merge, overlay of magenta and cyan or magenta, yellow and cyan channels. Scale bars, 5 µm.

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1. Birnbaum, J. *et al.* A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites. *Science (1979).* **367**, 51–59 (2020).
2. Abramson, J. *et al.* Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature 2024 630:8016* **630**, 493–500 (2024).
3. Xu, J. & Zhang, Y. How significant is a protein structure similarity with TM-score = 0.5? *Bioinformatics* **26**, 889 (2010).