Expanded View Figures

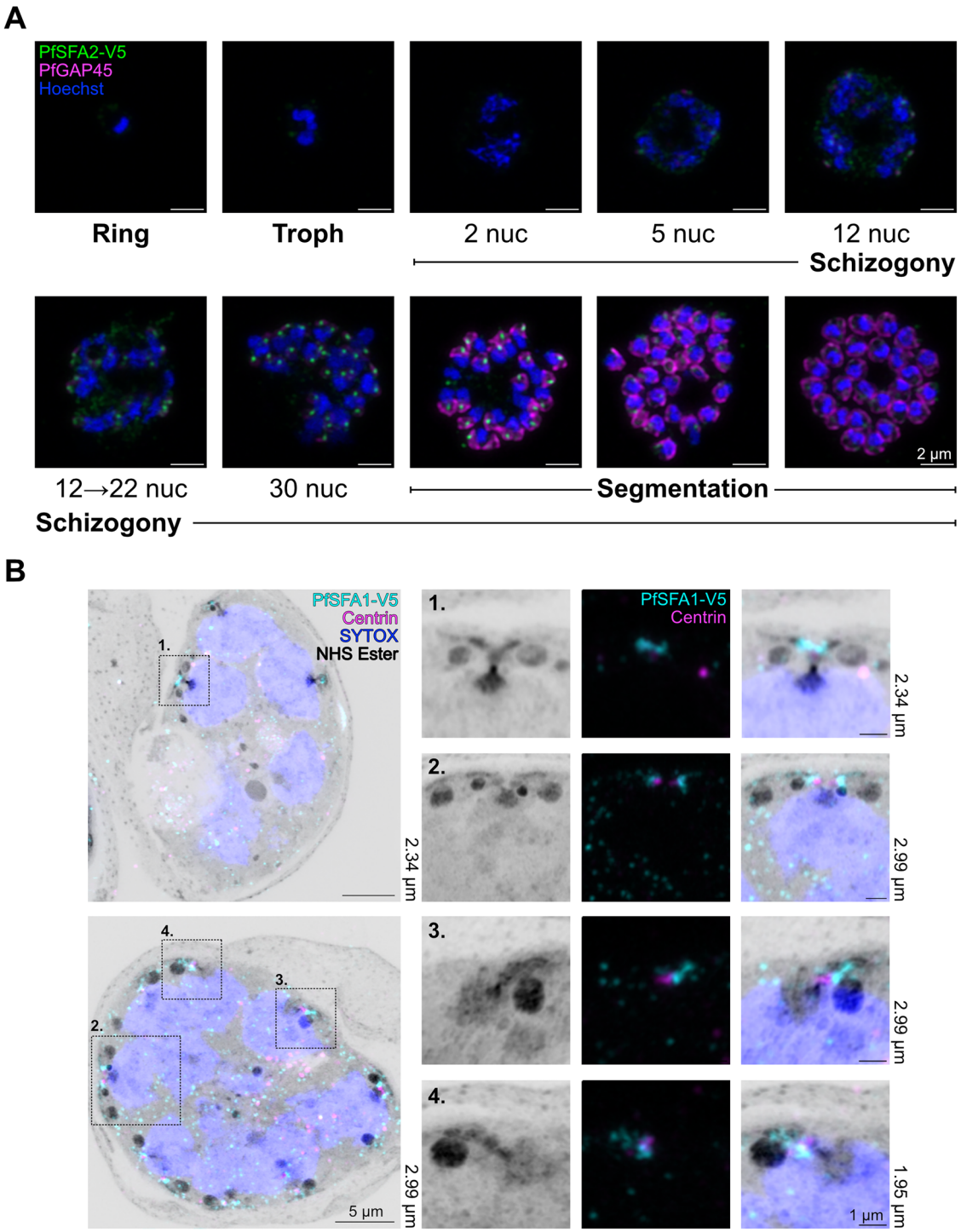

Figure EV1. Blood-stage immunofluorescence expression profile of PfSFA2 and ultrastructure expansion microscopy (U-ExM) of PfSFA1 localization.

(**A**) IFA of PfSFA2-smV5 expression across the asexual blood stages, stained with an anti-V5 antibody (green), an anti-PfGAP45 antibody (magenta), and Hoechst (DNA, blue). "Ring" stage with one nucleus, trophozoite stage ("Troph") with 2, and several stages throughout schizogony, including segmentation, are shown with the number of nuclei. The minimum and maximum displayed values for the PfSFA2-V5 and PfGAP45 channels follow the "Reset" values of the first segmentation image. Scale bars: 2 µm. (**B**) U-ExM of PfSFA1-smV5 schizonts stained with an anti-V5 antibody (cyan), an anti-CrCen antibody (outer-CP, magenta), SYTOX (DNA, blue), and an NHS ester-conjugated dye (grayscale). Sub-z-slices of a 7-nuclei (top) and a 14-nuclei (bottom) schizont are maximally projected and shown to the left. Representative centriolar plaque regions are numbered and shown as insets to the right. Z-depth of each maximum projection is shown to the right. Scale bars: 5 µm for full schizont; 1 µm for inset.

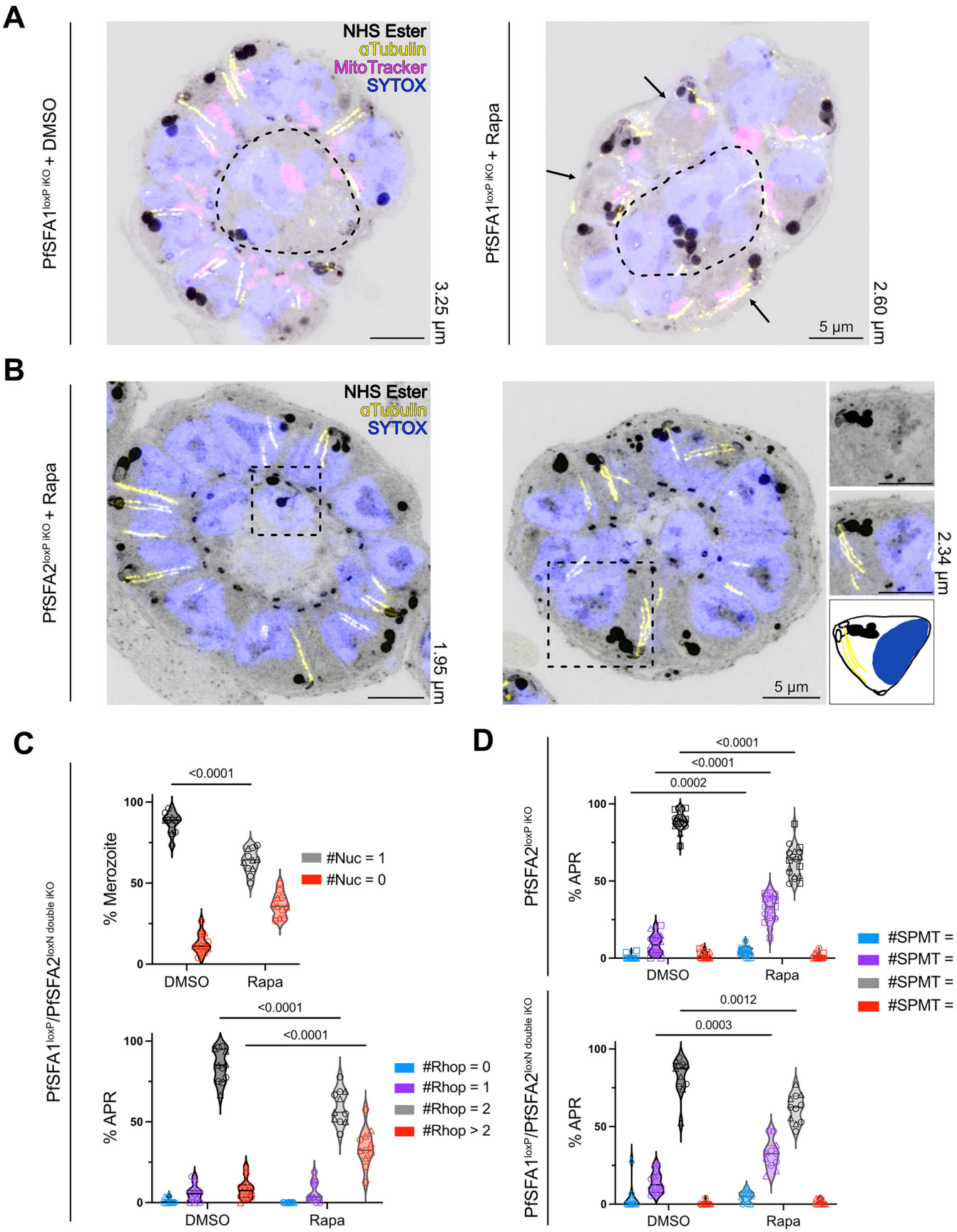
A
PfSFA1loxP iKO + DMSO
NHS Ester
αTubulin
MitoTracker
SYTOX
3.25 µm
PfSFA1loxP iKO + Rapa
5 µm
2.60 µm
B
PfSFA2loxP iKO + Rapa
NHS Ester
αTubulin
SYTOX
1.95 µm
5 µm
2.34 µm
C
PfSFA1loxP/PfSFA2loxN double iKO
% Merozoite
<0.0001
#Nuc = 1
#Nuc = 0
DMSO
Rapa
% APR
<0.0001
<0.0001
#Rhop = 0
#Rhop = 1
#Rhop = 2
#Rhop > 2
D
PfSFA2loxP iKO
% APR
<0.0001
<0.0001
0.0002
#SPMT = 0
#SPMT = 1
#SPMT = 2
#SPMT = 3
PfSFA1loxP/PfSFA2loxN double iKO
0.0012
0.0003

Figure EV2. Additional PfSFA1 and/or PfSFA2 inducible knockout (iKO) phenotypes and quantifications.

(**A**) U-ExM of segmented PfSFA1$^{loxP\ iKO}$ schizonts (with C-terminal 2xMyc tag) incubated with DMSO (control) or Rapa (KO) and stalled by Compound-1. Representative maximum projection images are shown. Parasites are stained with NHS ester-conjugated dye (grayscale), an alpha-Tubulin antibody (yellow), MitoTracker (magenta), and SYTOX (DNA, blue). Residual bodies are highlighted with black dashed lines. Arrows indicate anucleate merozoites. Minimum and maximum display values of the NHS channel are manually adjusted to maximize clarity of parasite structures. Scale bars: 5 µm. (**B**) U-ExM of two segmented PfSFA2$^{loxP\ iKO}$ schizonts incubated with Rapa (KO) and stalled by Compound-1. Representative maximum projection images are shown. Parasites are stained with NHS ester-conjugated dye (grayscale), an alpha-Tubulin antibody (yellow), and SYTOX (DNA, blue). Detached rhoptries in the residual body are highlighted in the left image. A merozoite with two APRs is highlighted and shown in insets in the right image. Minimum and maximum display values of the NHS channel are manually adjusted to maximize clarity of parasite structures. Scale bars: 5 µm. (**C**) Nucleus and rhoptry phenotypes in the control (DMSO) or PfSFA1/PfSFA2 double KO (Rapa) conditions are quantified for PfSFA1^{loxP}/PfSFA2$^{loxN\ double\ iKO}$ schizonts. First violin plot displays the distribution of the percentage of merozoites with (#Nuc = 1) or without nucleus (#Nuc = 0) per schizont for the two conditions. The second violin plot shows the distribution of the percentage of APRs associated with 0, 1, 2, or >2 rhoptries (#Rhop) per schizont for each condition. Each data point represents a single schizont (5–6 per biological replicate, 11 total per condition), pooled across two biologically independent experiments. Data points from different biological replicates are represented by different shapes (circles and triangles). Statistical significance was determined by the Mann–Whitney test (with multiple testing correction using Benjamini, Krieger, and Yekutieli false discovery rate method) for nuclear quantification and by two-way repeated measures ANOVA (with post hoc Šídák's multiple-comparisons test) for rhoptry quantification. Only the uncorrected *p* value for the nucleated phenotype is plotted on the graph for clarity, and only significant *p* values are plotted in general. For each violin, the mean is indicated by a solid line, and the first and third quartiles are shown as dashed lines. (**D**) Number of subpellicular microtubules (SPMTs) associated with each apical polar ring (APR) within a schizont are quantified for both PfSFA2$^{loxP\ iKO}$ schizonts (left violin plot) and PfSFA1^{loxP}/PfSFA2$^{loxN\ double\ iKO}$ schizonts (right violin plot). Each plot shows the distribution of the percentage of APRs associated with 0, 1, 2, or 3 SPMTs (#SPMT) per schizont for the control (DMSO) or KO (Rapa) condition. Each data point represents a single schizont pooled across three (for PfSFA2$^{loxP\ iKO}$) or two (for PfSFA1^{loxP}/PfSFA2$^{loxN\ double\ iKO}$) biologically independent experiments. Data points from different biological replicates are represented by different symbols. Statistical significance was determined by two-way repeated measures ANOVA with post-hoc Šídák's multiple-comparisons test. Only significant *p* values are plotted. For each violin, the mean is shown as a solid line and the first and third quartiles are shown as dashed lines. Z-depth of maximum projection is shown at the right of each image/panel.

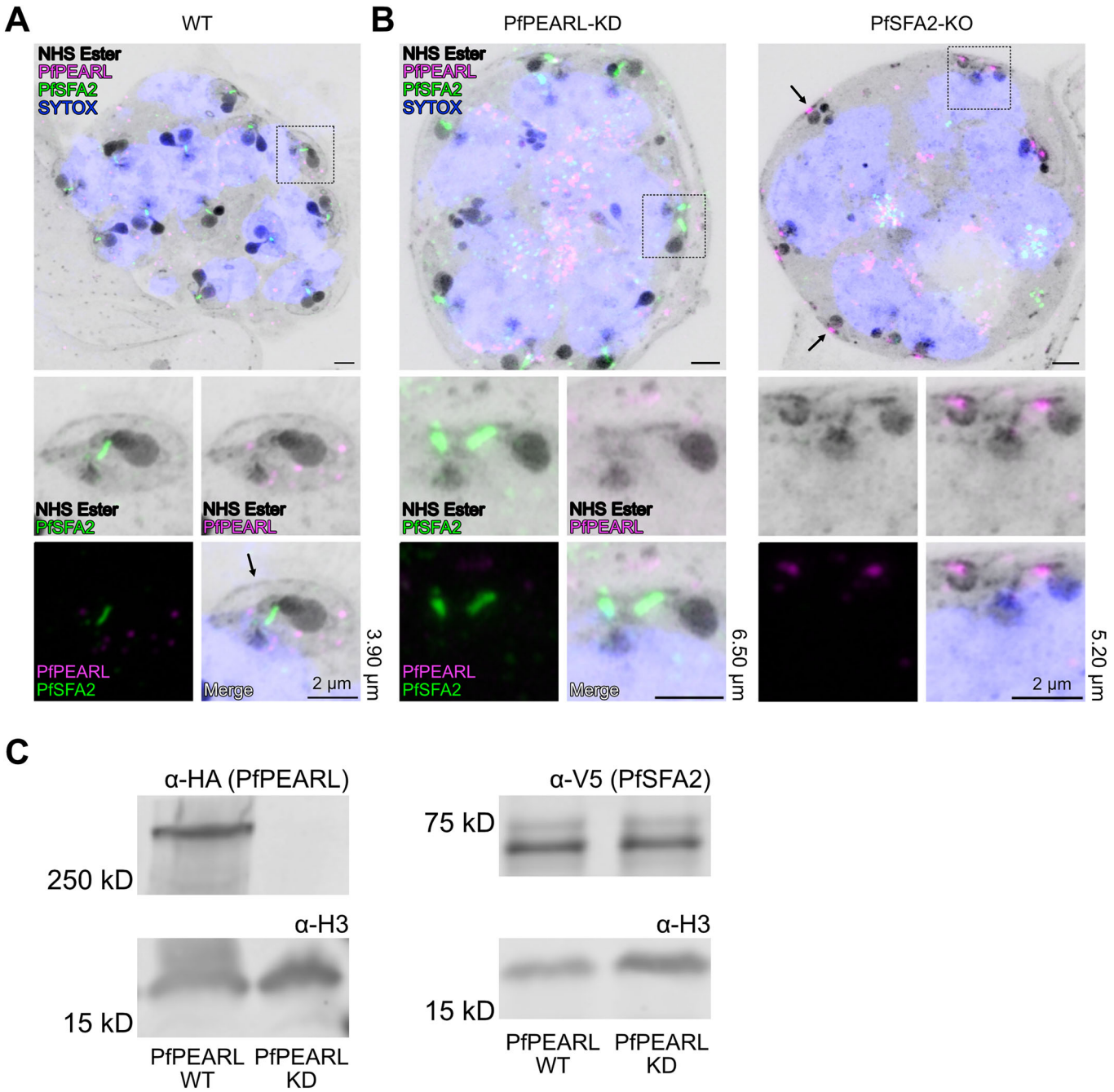

Figure EV3. PfPEARL localization disappears before PfSFA2, and the two localize independently of each other.

(**A**, **B**) U-ExM localization of PfPEARL-smHA and PfSFA2-smV5 in WT (**A**), PfPEARL-KD and PfSFA2-KO (**B**) conditions visualized with NHS ester-conjugated dye (grayscale), an anti-HA antibody (magenta), an anti-V5 antibody (green), and SYTOX (DNA, blue). Inset in (**A**) shows an example of a segmenting merozoite with an arrow pointing to the basal complex. Inset below each schizont in (**B**) shows a dividing nucleus-apical pole region. Arrows point to apical poles disconnected from a centriolar plaque in the PfSFA2-KO schizont. Scale bars: 2 µm. Inset below each schizont shows a dividing nucleus-apical pole region. Arrows point to apical poles disconnected from a centriolar plaque in the PfSFA2-KO schizont. Scale bars: 2 µm. (**C**) Western blots of PfPEARL WT and KD conditions stained with an anti-HA antibody for PfPEARL-smHA protein level (left, >250 kD band) and an anti-V5 antibody for PfSFA2-smV5 protein level (right, ~75 kD band). Both blots were co-stained with an anti-H3 antibody for histone H3 protein level (~15 kD band) to normalize between pellets. Z-depth of maximum projection is shown at the right of each image/panel.

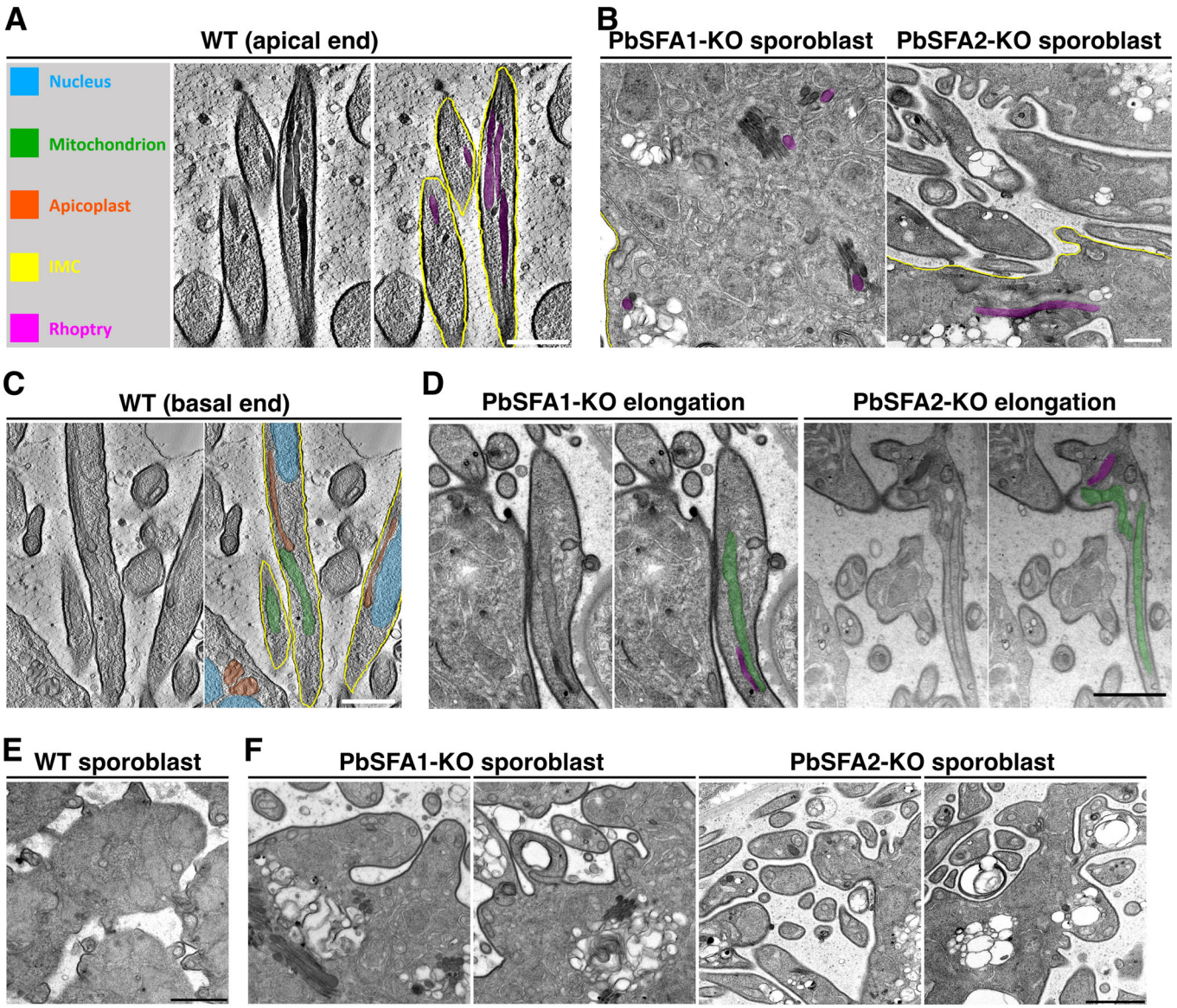

Figure EV4. Organelle formation and positioning are dysregulated in PbSFA1-KO and PbSFA2-KO parasites.

(**A**) Original and colored slices through electron tomograms showing dense, elongated rhoptries positioned at the apical end of WT sporozoites. Labels: rhoptries, magenta and IMC, yellow. Scale bar: 1 µm. (**B**) Original and colored transmission electron micrographs of PbSFA1-KO and PbSFA2-KO oocysts showing dense, elongated rhoptries (partly labeled in magenta) remained in sporoblast. Scale bar: 500 nm. (**C**) Original and colored slices through electron tomograms showing apicoplast in close proximity to the nucleus and mitochondrion placed behind. Labels: nuclei, blue; IMC, yellow; apicoplast, orange; and mitochondria, green. Scale bar: 1 µm. (**D**) Original and colored transmission electron micrographs of budding elongations in PbSFA1-KO (left) and PbSFA2-KO (right) oocysts with mitochondria in elongation and rhoptries in close proximity. Scale bars: 1 µm. (**E**) Electron micrograph of sporoblasts from a WT oocyst showing IMC is only restricted at the area of newly formed sporozoites. Scale bar: 2 µm. (**F**) Electron micrographs of sporoblasts from PbSFA1-KO and PbSFA2-KO oocysts showing IMC enveloping the entire sporoblast. Scale bar: 1 µm.

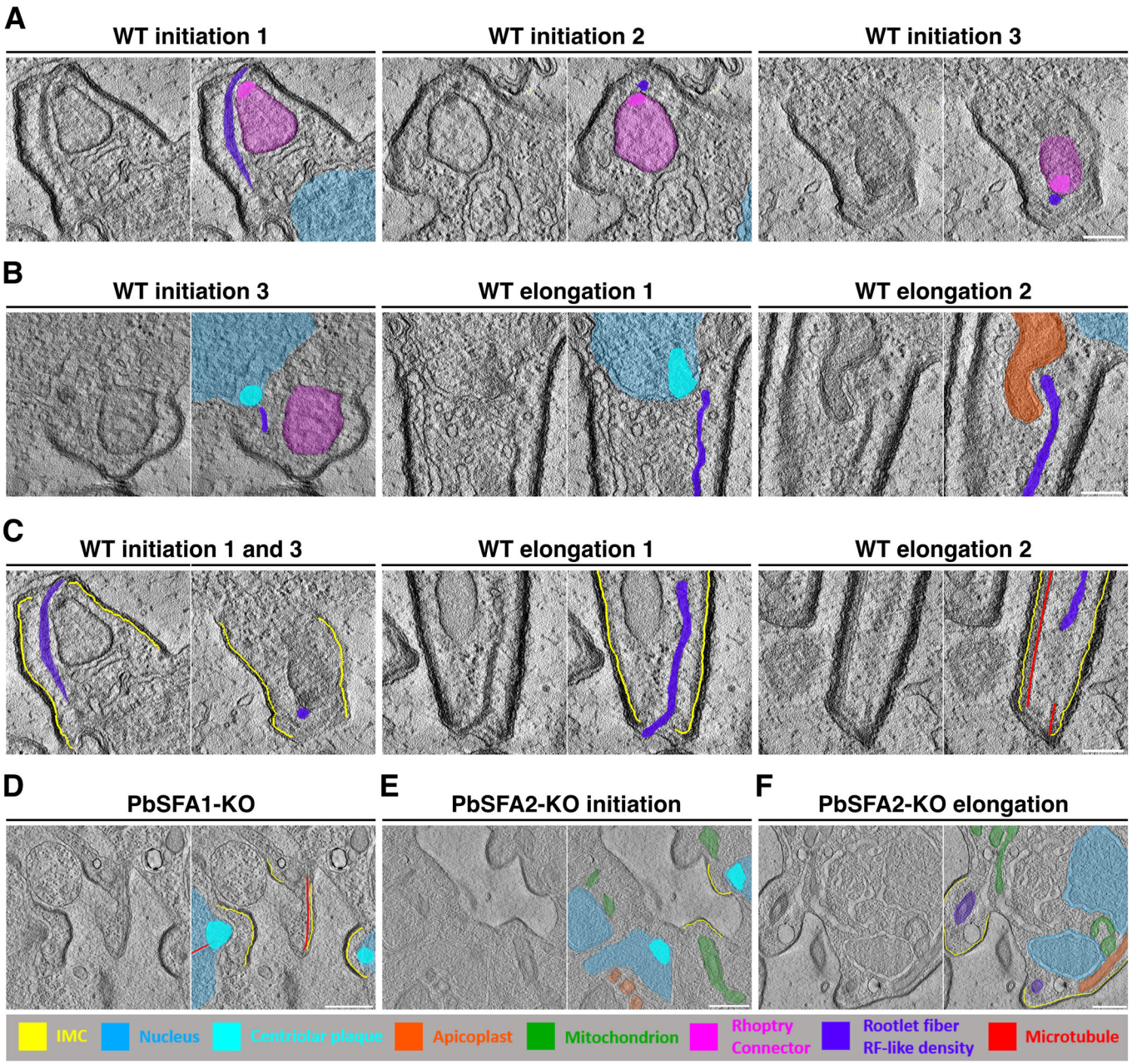

Figure EV5. Details of the rootlet fiber and associated organelles resolved by electron tomography.

(**A**) Original and colored slices through electron tomograms showing three WT parasites at the initial budding stage. The rootlet fibers were in close proximity to the densities in rhoptry anlagen from side view (initiation 1) and top views (initiation 2–3). Scale bars: 200 nm. (**B**) Original and colored slices through electron tomograms showing one WT parasite at the initial budding stage and two WT parasites at the elongated budding stage. The rootlet fibers were in close proximity to a centriolar plaque (initiation 3, elongation 1) and apicoplast (elongation 2). Scale bars: 200 nm. (**C**) Original and colored slices through electron tomograms showing two WT parasites at the initial budding stage (same slices as panel A but colored differently, only colored slices were shown) and two WT parasites at the elongated budding stage. The tips were almost flat, likely due to the presence of the apical polar ring, where the subpellicular microtubules are initiated from. Scale bars: 200 nm. (**D**) Original and colored slices through electron tomograms showing the initial buds and elongations of PbSFA1-KO parasites (same sample shown at a different z-slice (z = 117) and highlighted differently than Fig. 8C (z = 114)). No rootlet fiber or rhoptry anlage appeared in the sporozoite buds, and the tips of the buds appear dome-shaped. Scale bars: 500 nm. (**E**) Original and colored slices through electron tomograms showing the initial buds of PbSFA2-KO parasites (same sample shown at a different z-slice (z = 110) and highlighted differently than Fig. 8E (z = 108)). No rootlet fiber or rhoptry anlage appeared in the sporozoite buds, and the tips of the buds appear dome-shaped like PbSFA1-KO. Scale bars: 500 nm. (**F**) Original and colored slices through electron tomograms showing the budding elongations of PbSFA2-KO parasites. No rootlet fiber or rhoptry anlage appeared in the sporozoite buds, but additional thick and striated densities were visible in the elongation of both buds. Scale bars: 500 nm. Labels: IMC, yellow; nuclei, blue; centriolar plaques, cyan; apicoplasts, orange; mitochondria, green; rhoptry anlagen and the connector to the fibers, magenta; rootlet fibers and RF-like densities in PbSFA2-KO, purple; microtubules, red.