Enzymes involved in plastid-targeted phosphatidic acid synthesis are essential for *Plasmodium yoelii* liver stage development

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Creation of transgenic *P. yoelii* parasites bearing C-terminal 4xmyc epitope tags. Epitope tags were created on (A) PY00789 (apiG3PDH) (B) *P. yoelii* apiG3PAT, (C) PY01678 (LPAAT?), and (D) PY02486 (LPAAT?). A schematic of the genomic locus of the wildtype and transgenic parasite is annotated with the primer pairs (half arrows) used for genotyping PCR and their resulting product sizes. Genomic regions used for recombination are denoted with crossed lines between the wildtype and transgenic loci. The molecular weight marker used was the 1kb DNA Ladder from New England Biolabs.

Supplemental Figure 2: *P. yoelii* apiG3PDHmyc and apiG3PATmyc are not expressed in blood stage parasites. Wildtype (WT) and epitope tagged parasite blood stages were subjected to immunofluorescent assay (IFA). The apicoplast was visualized using an antibody to the acyl carrier protein (ACP), the endoplasmic reticulum (ER) using an antibody to binding immunoglobulin protein (BiP) and the tagged protein using an antibody to myc. WT blood stage parasites, as expected, express ACP and BiP (A) but do not express myc (B). apiG3PDHmyc parasites (C) and apiG3PATmyc parasites (D) express ACP but not myc. DNA was stained with 4',6-diamidino-2-phenylindole. Scale bar: 10 μm.

Supplemental Figure 3: Supplemental Figure 2: *P. yoelii* apiG3PDHmyc and apiG3PATmyc are not expressed in salivary gland sporozoites. Wildtype (WT) and epitope tagged salivary gland sporozoites

were subjected to immunofluorescent assay (IFA). The apicoplast was visualized using an antibody to the acyl carrier protein (ACP), the sporozoite surface using an antibody to circumsporozoite protein (CSP) and the tagged protein using an antibody to myc. WT salivary gland sporozoites, as expected, express ACP and CSP (A) but do not express myc (B). apiG3PDHmyc parasites (C) and apiG3PATmyc parasites (D) express ACP but not myc. DNA was stained with 4',6-diamidino-2-phenylindole. Scale bar: 10 μm.

Supplemental Figure 4: Epitope tag expression in transgenic *P. yoelii* blood stage parasites. Equal amounts of blood stage parasite lysate was subjected to Western blotting after SDS polyacrylamide electrophoresis and probed with an antibody to myc. The results show that wildtype parasites (WT) do not express a non-specific protein with a myc epitope. No myc epitope-tagged *P. yoelii* apiG3PDHmyc, apiG3PATmyc or PY02486myc were detected. As predicted, based on our IFA results with the *PY01678myc* parasite, a strong myc signal for the predicted size of PY01678myc (approximately 38 kDa) was seen.

Supplemental Figure 5: Salivary gland sporozoite production in the mosquito is not affected in the *P. yoelii apiG3PDHmyc*, *apiG3PATmyc*, *PY01678myc*, *PY02486myc*, *apiG3PDH*(-) and *apiG3PAT*(-) parasite. Salivary gland sporozoite enumeration was averaged from at least 20 dissected mosquitoes 14 days after the infectious blood meal. Enumeration was assessed from four independent infections and the results are presented as the average number of sporozoites per mosquito over the four independent infections +/- the standard deviation. Numbers are not statistically different.

Supplemental Figure 6: The *Plasmodium* glycerol 3-phosphate acyl transferase (apiG3PAT) predicted to target to the apicoplast is expressed during liver stages and co-localizes with the apicoplast lumen-targeted acyl carrier protein (ACP). The expression of apiG3PAT was assessed with the transgenic epitope-tagged *Py apiG3PATmyc* parasite and identified by IFA. At 24 hours (A), nuclear replication was apparent (yellow arrows point to multiple nuclei centers) and apiG3PAT expression was

in internal structures reminiscent of the apicoplast. Antibody to circumsporozoite protein (CSP) was used to delineate the parasite plasma membrane (PPM). At 30 hours (B), liver stage parasites had increased in size and it was clear that apiG3PAT perfectly co-localized with the apicoplast lumen protein acyl carrier protein (ACP) and apiG3PAT localization was contained within the parasitophorous vacuole membrane (PVM) marker Hep17 (C). At 48 hours (D), co-localization of apiG3PAT with ACP clearly shows that apiG3PAT is localized to the extensively branched apicoplast in a late-liver stage parasite. Fully mature liver stages at 48 hours showed the presence of merozoites, localized with the PPM marker merozoite surface protein 1 (MSP1) and each merozoite, as expected, appeared to contain a single apicoplast, based on apiG3PAT expression (E). DNA was stained with 4',6-diamidino-2-phenylindole. Scale bar: 10 μm.

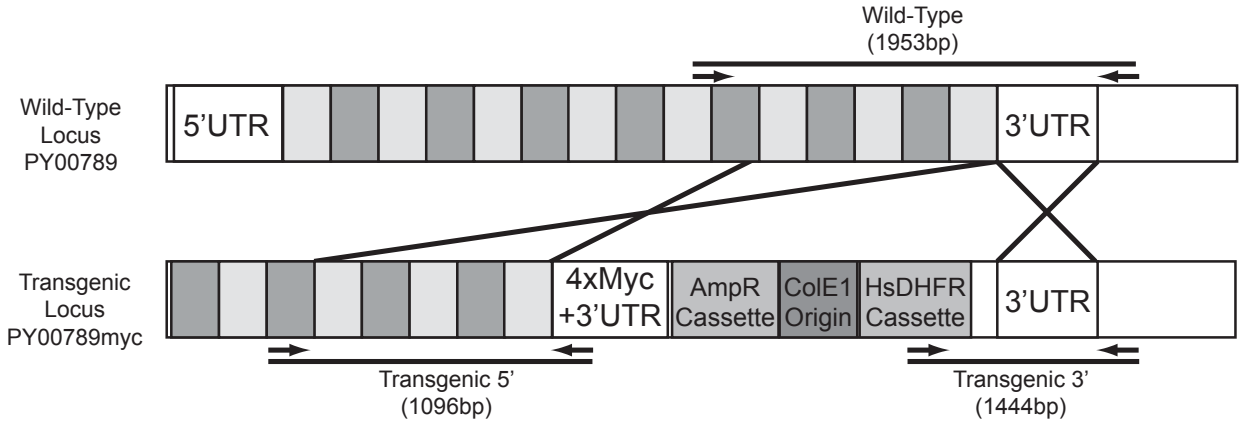
Supplemental Figure 7: Creation of transgenic *P. yoelii* knockout parasites. Gene knockouts were attempted and/or created for (A) PY00789 (apiG3PDH), (B) *P. yoelii* apiG3PAT, and (C) PY01678 (LPAAT?). A schematic of the genomic locus of the wildtype and transgenic parasites is annotated with the primer pairs (half arrows) used for genotyping PCR and their resulting product sizes. Genomic regions used for recombination are denoted with crossed lines between the wildtype and transgenic loci. The molecular weight marker used was the 1kb DNA Ladder from New England Biolabs.

Supplemental Figure 8. *P. yoelii* blood stage replication is not affected by loss of *apiG3PDH* or *apiG3PAT*. One million *P. yoelii* wildtype (WT), *Py apig3pdh*(-) (clone 1) and *Py apig3pat*(-) (clone 1) blood stage parasites were injected into groups of five Swiss Webster mice. Blood stage parasitemia was determined daily for seven days by Giemsa-stained thin blood smear. Results are presented as the average between the groups of five mice. There is no statistical difference between the growth rates.

Supplemental Figure 9: Gene knockout of *P. yoelii* apicoplast-targeted glycerol 3-phosphate acyltransferase (apiG3PAT) results in arrest in liver stage development. Loss of *apiG3PAT* was assessed with the *Py apig3pat*(-) parasite by IFA. At 24 hours (A), liver stage development appeared

normal, based on expression of the parasite plasma membrane (PPM)-localized circumsporozoite protein (CSP) and the apicoplast-targeted acyl carrier protein (ACP) and parasite size (compare to Fig. S2A). At 30 hours (B), nuclear replication and apicoplast development were retarded compared to wildtype parasites although the parasitophorous vacuole membrane (PVM), based on Hep17 localization, appeared intact (compare to Fig. S2C). At 48 hours (C), little or no MSP1 expression was seen in *Py apig3pat*(-) liver stages (compare to Fig. S2E). Additionally, the liver stages were smaller and both nuclear replication and apicoplast complexity were stunted. At 65 hours (D), *Py apig3pat*(-) liver stages were detected, the majority of which were expressing MSP1 but not ACP, suggesting that apicoplast integrity was compromised. DNA was stained with 4',6-diamidino-2-phenylindole. Scale bar: 10 μm.

FIGURE S1A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(1953bp)
Wild-Type
Locus
PY00789
5'UTR
3'UTR
Transgenic
Locus
PY00789myc
4xMyc
+3'UTR
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(1096bp)
Transgenic 3'
(1444bp)

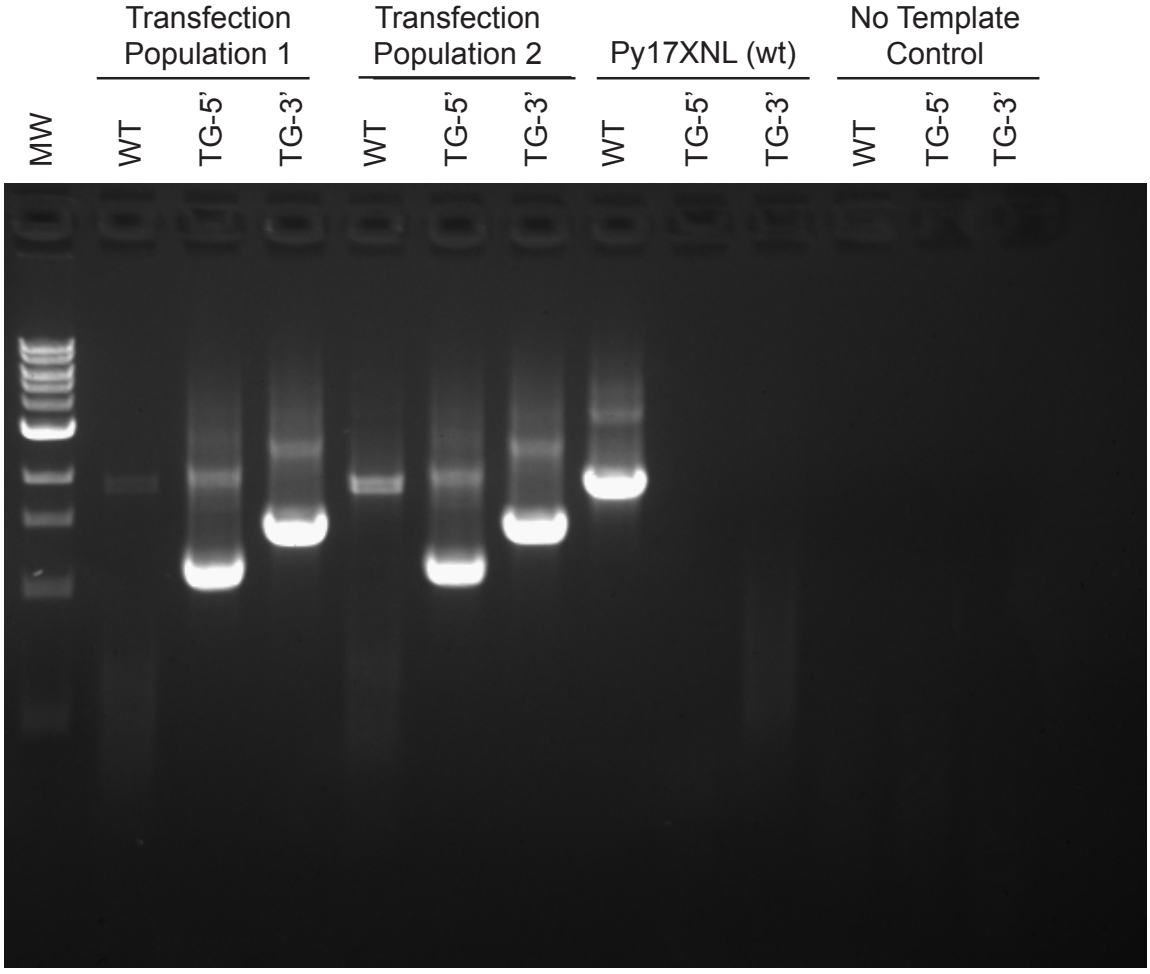
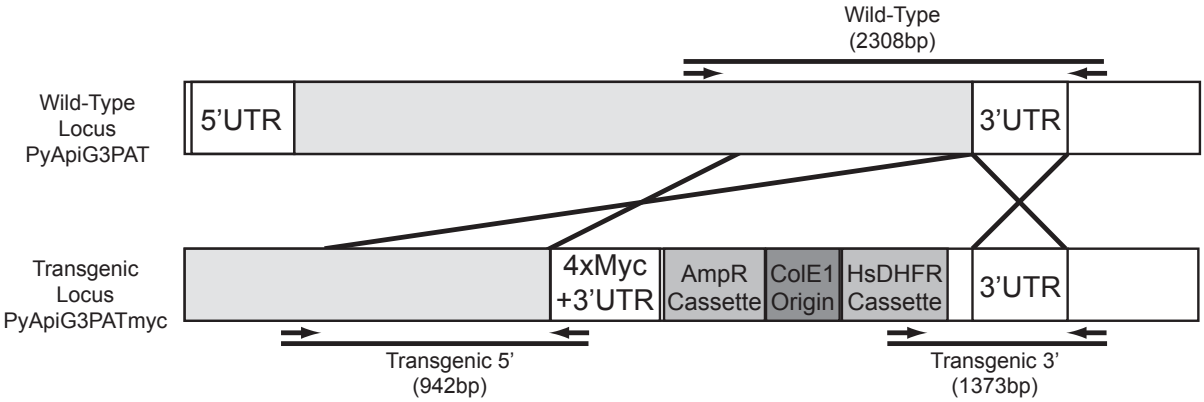

Transfection
Population 1
Transfection
Population 2
Py17XNL (wt)
No Template
Control
MW
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'

FIGURE S1B


Wild-Type
(2308bp)
Wild-Type
Locus
PyApiG3PAT
5'UTR
3'UTR
Transgenic
Locus
PyApiG3PATmyc
4xMyc
+3'UTR
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(942bp)
Transgenic 3'
(1373bp)

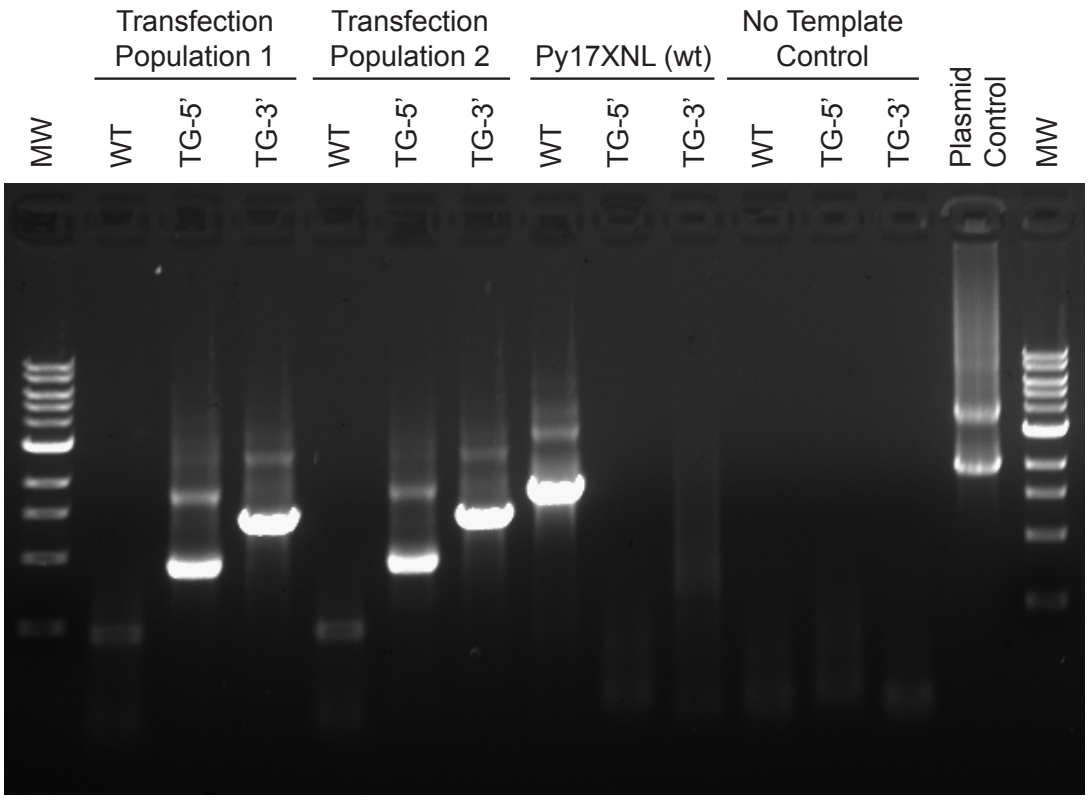
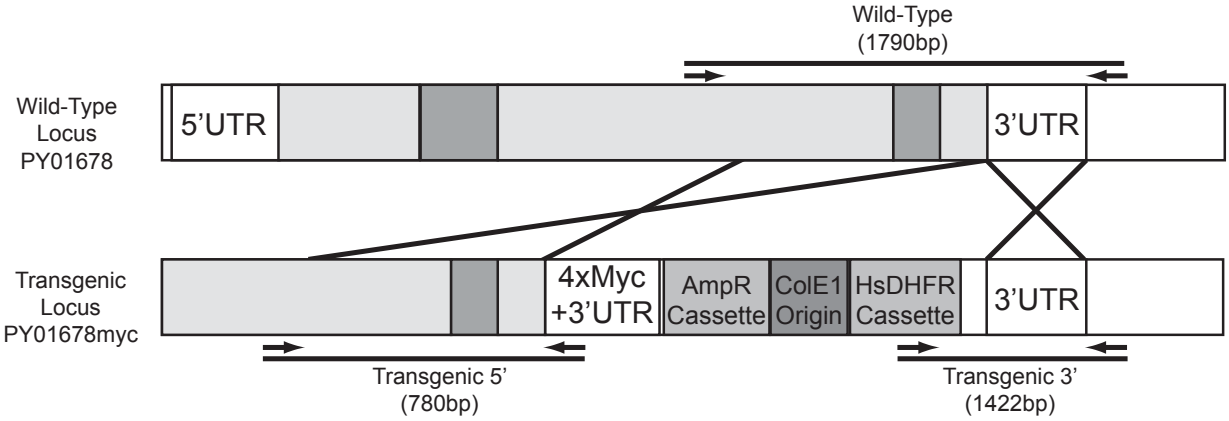

Transfection
Population 1
Transfection
Population 2
Py17XNL (wt)
No Template
Control
MW
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
Plasmid
Control
MW

FIGURE S1C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(1790bp)
Wild-Type
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5'UTR
3'UTR
Transgenic
Locus
PY01678myc
4xMyc
+3'UTR
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(780bp)
Transgenic 3'
(1422bp)

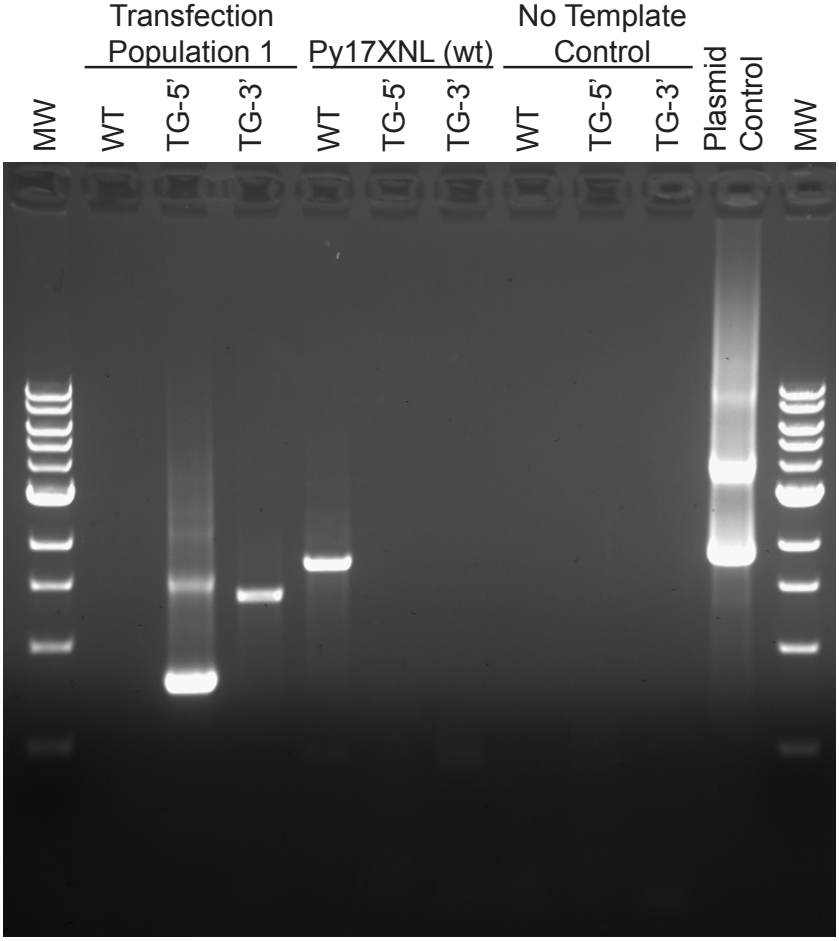

Transfection
Population 1
Py17XNL (wt)
No Template
Control
MW
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
WT
TG-5'
TG-3'
Plasmid
Control
MW

FIGURE S1D

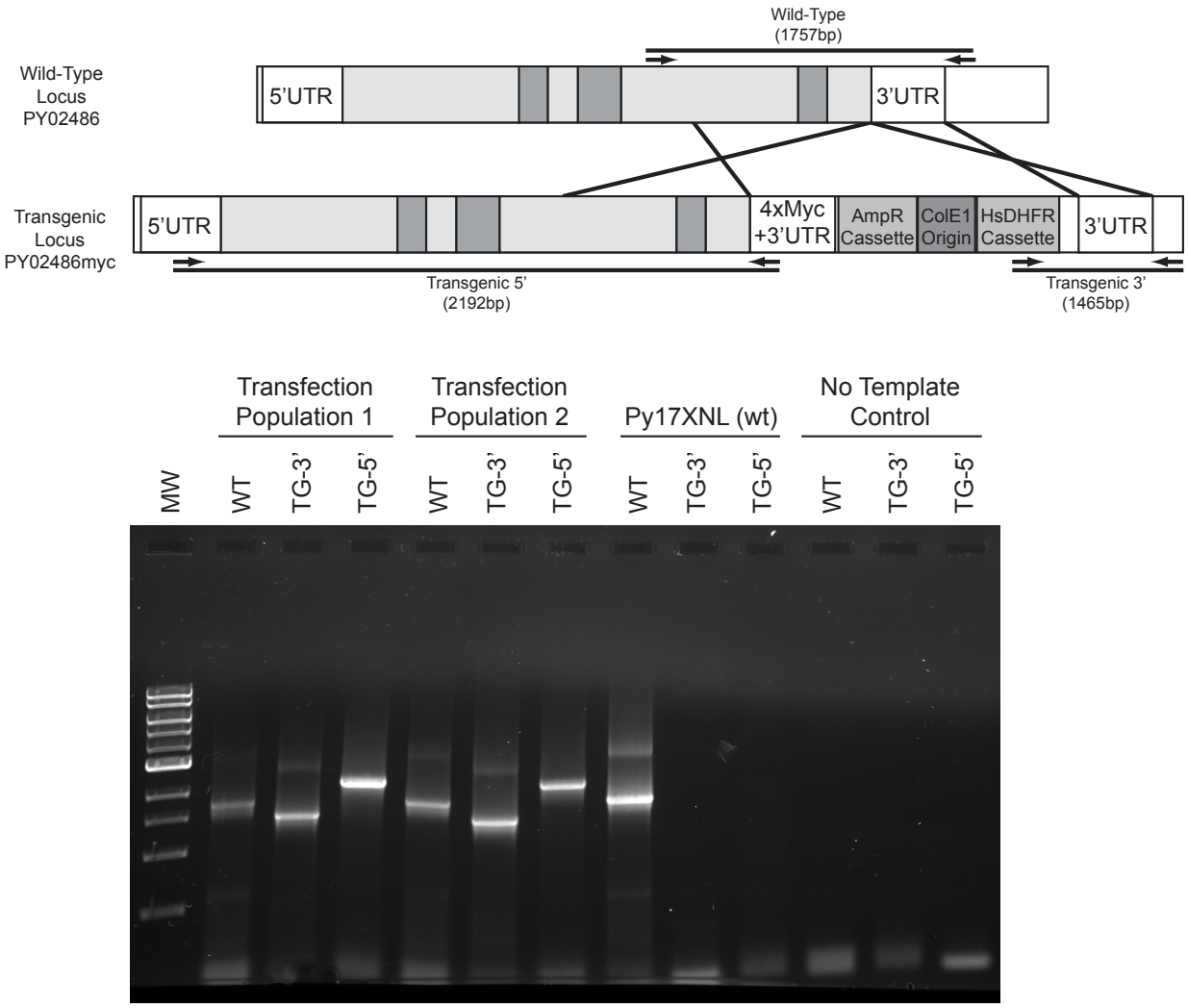

Wild-Type
(1757bp)
Wild-Type
Locus
PY02486
5'UTR
3'UTR
Transgenic
Locus
PY02486myc
5'UTR
4xMyc
+3'UTR
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(2192bp)
Transgenic 3'
(1465bp)
Transfection
Population 1
Transfection
Population 2
Py17XNL (wt)
No Template
Control
MW
WT
TG-3'
TG-5'
WT
TG-3'
TG-5'
WT
TG-3'
TG-5'
WT
TG-3'
TG-5'

FIGURE S2

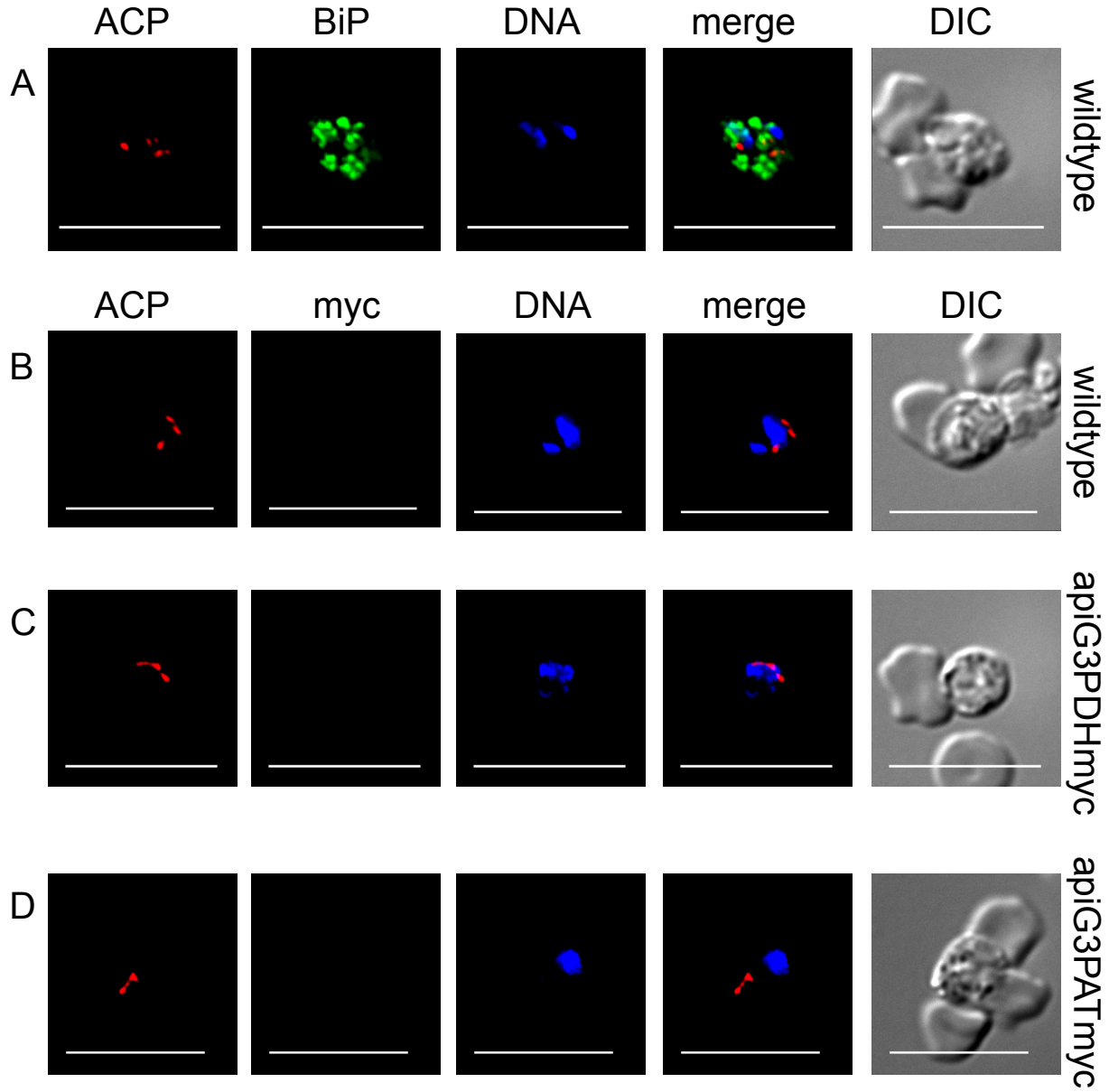

ACP
BiP
DNA
merge
DIC
A
wildtype
ACP
myc
DNA
merge
DIC
B
wildtype
C
apiG3PDHmyc
D
apiG3PATmyc

FIGURE S3

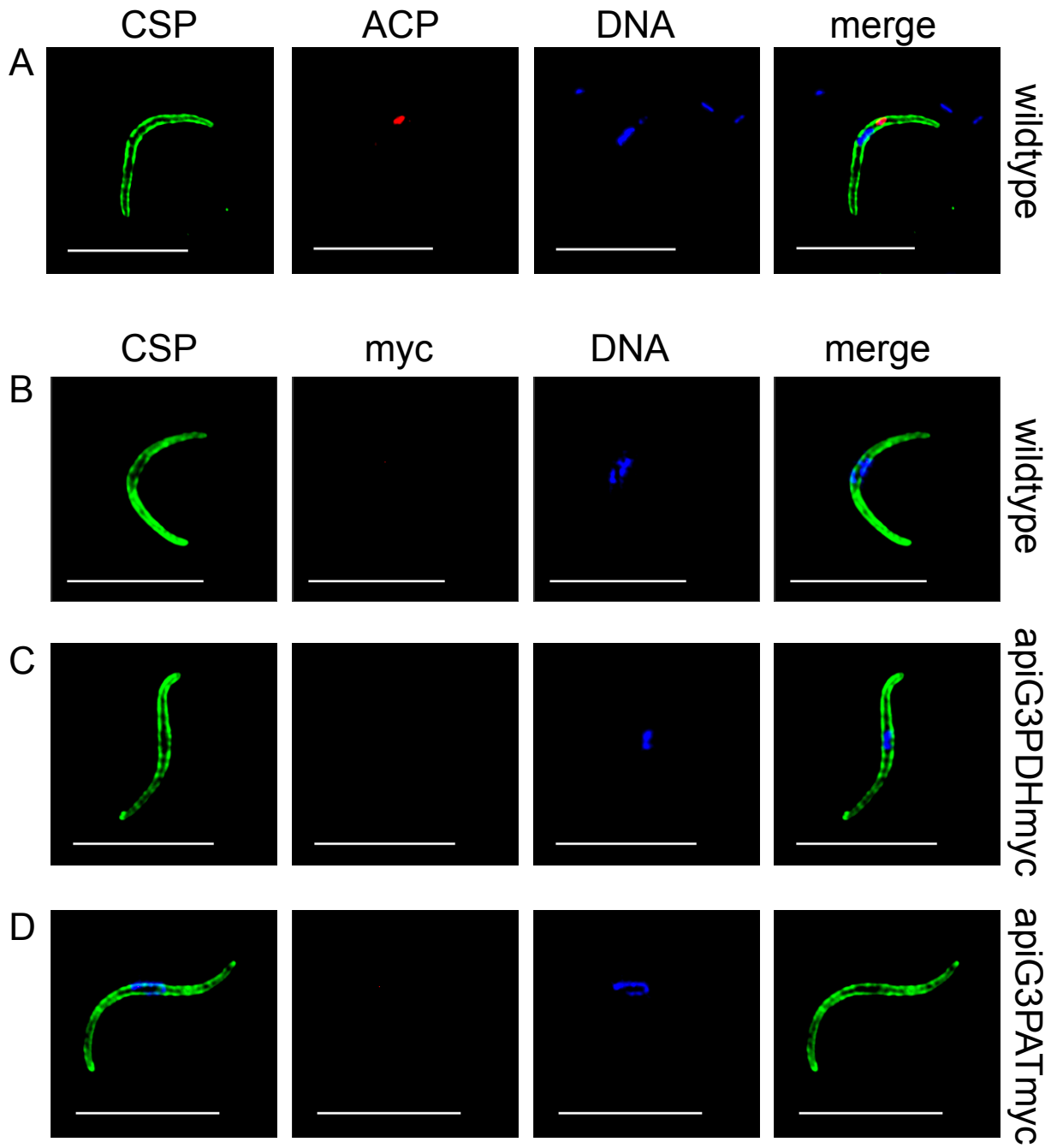

CSP
ACP
DNA
merge
A
wildtype
CSP
myc
DNA
merge
B
wildtype
C
apiG3PDHmyc
D
apiG3PATmyc

FIGURE S4

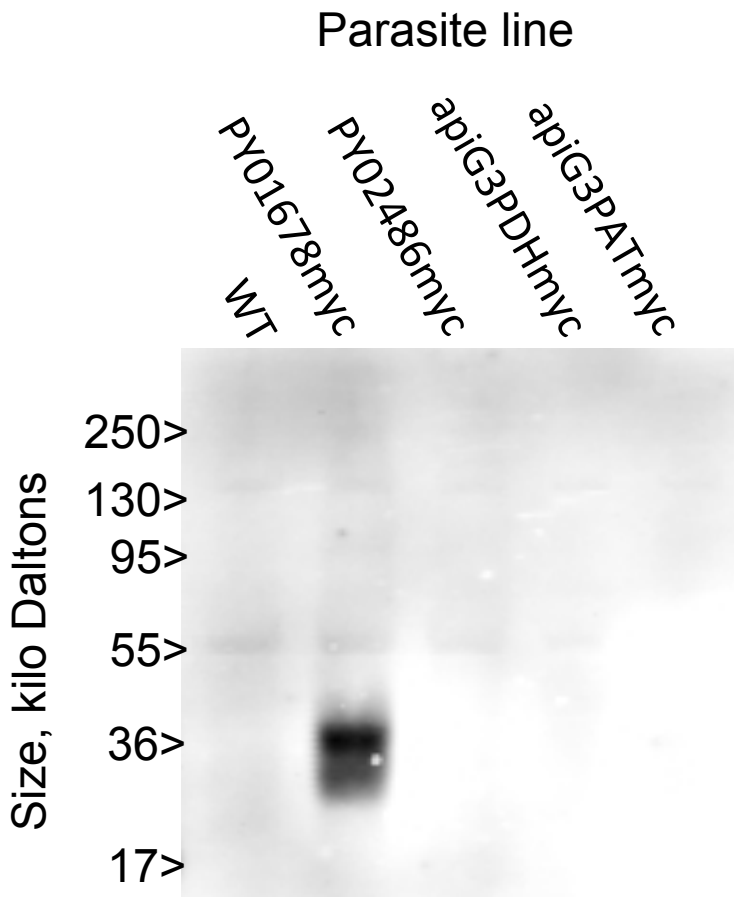

Parasite line
WT
PY01678myc
PY02486myc
apiG3PDHmyc
apiG3PATmyc
Size, kilo Daltons
250>
130>
95>
55>
36>
17>

FIGURE S5

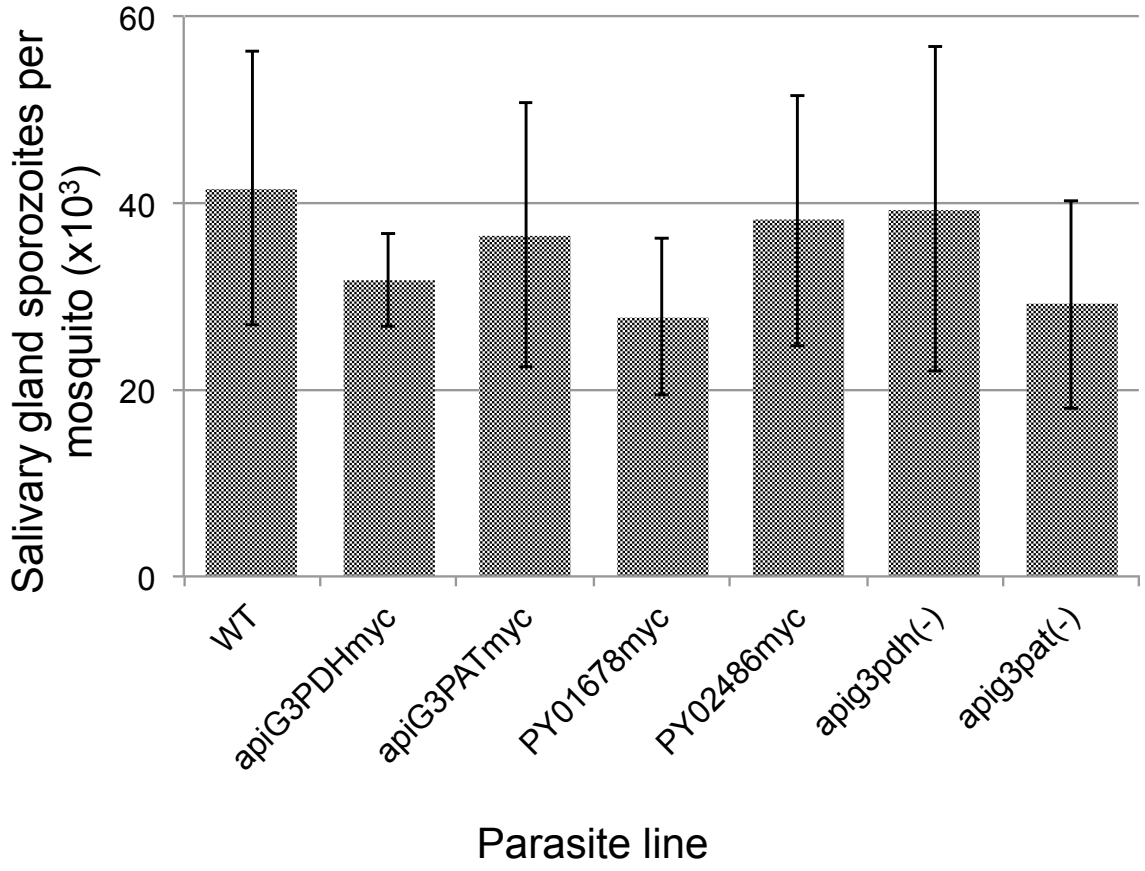

Salivary gland sporozoites per mosquito (x10³)
60
40
20
0
WT
apiG3PDHmyc
apiG3PATmyc
PY01678myc
PY02486myc
apig3pdh(-)
apig3pat(-)
Parasite line

FIGURE S6

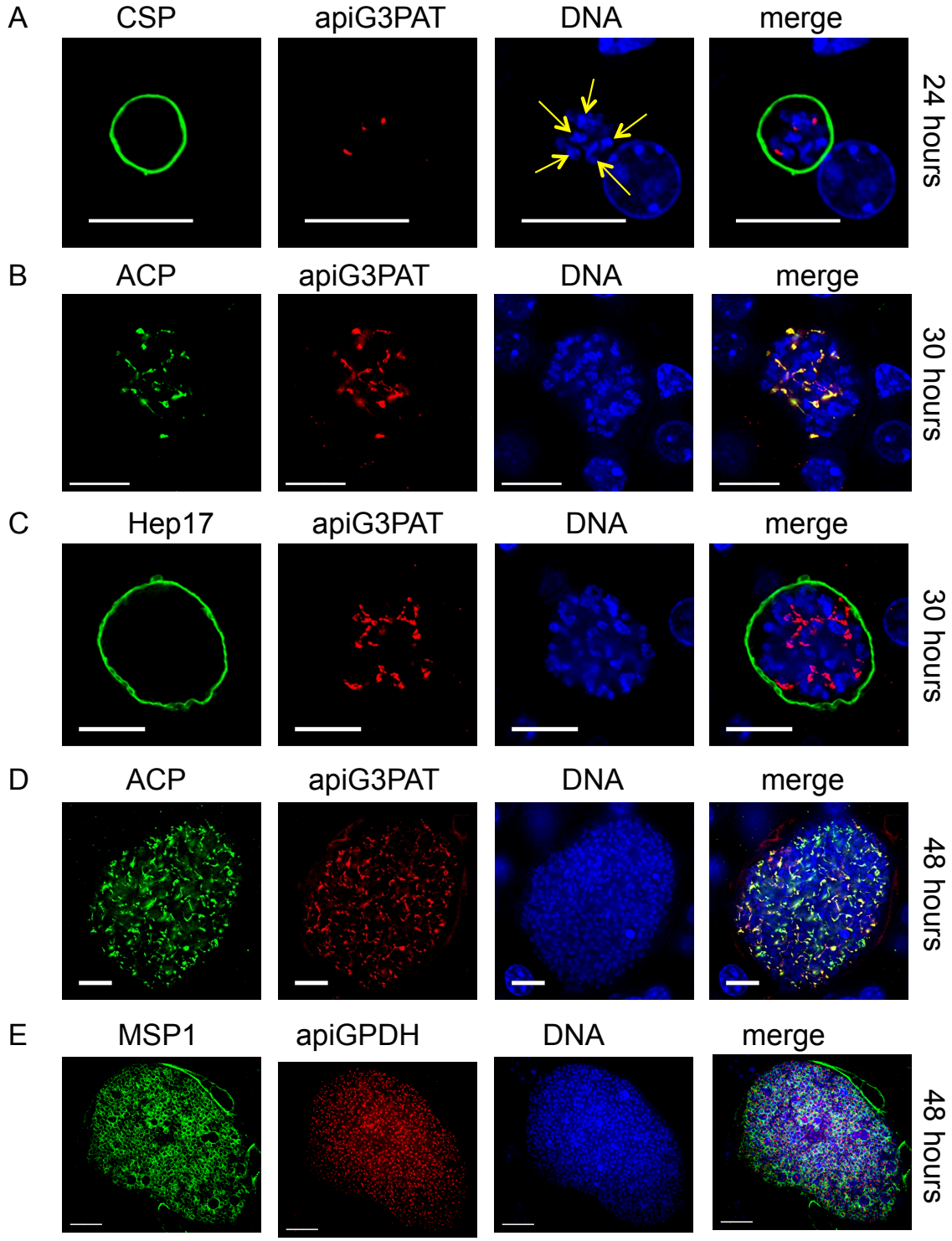
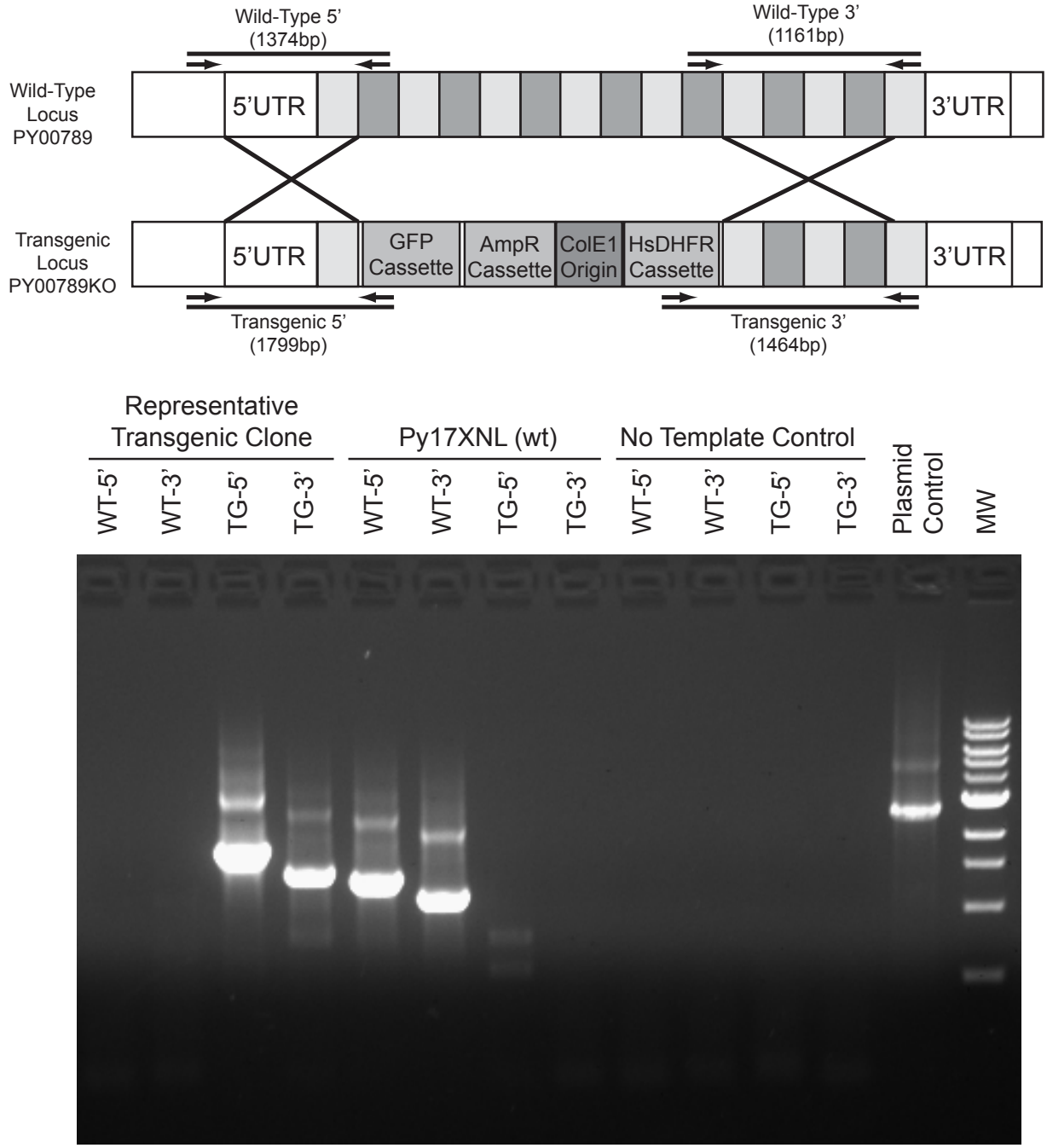

A
CSP
apiG3PAT
DNA
merge
24 hours
B
ACP
apiG3PAT
DNA
merge
30 hours
C
Hep17
apiG3PAT
DNA
merge
30 hours
D
ACP
apiG3PAT
DNA
merge
48 hours
E
MSP1
apiGPDH
DNA
merge
48 hours

FIGURE S7A


Wild-Type 5'
(1374bp)
Wild-Type 3'
(1161bp)
Wild-Type
Locus
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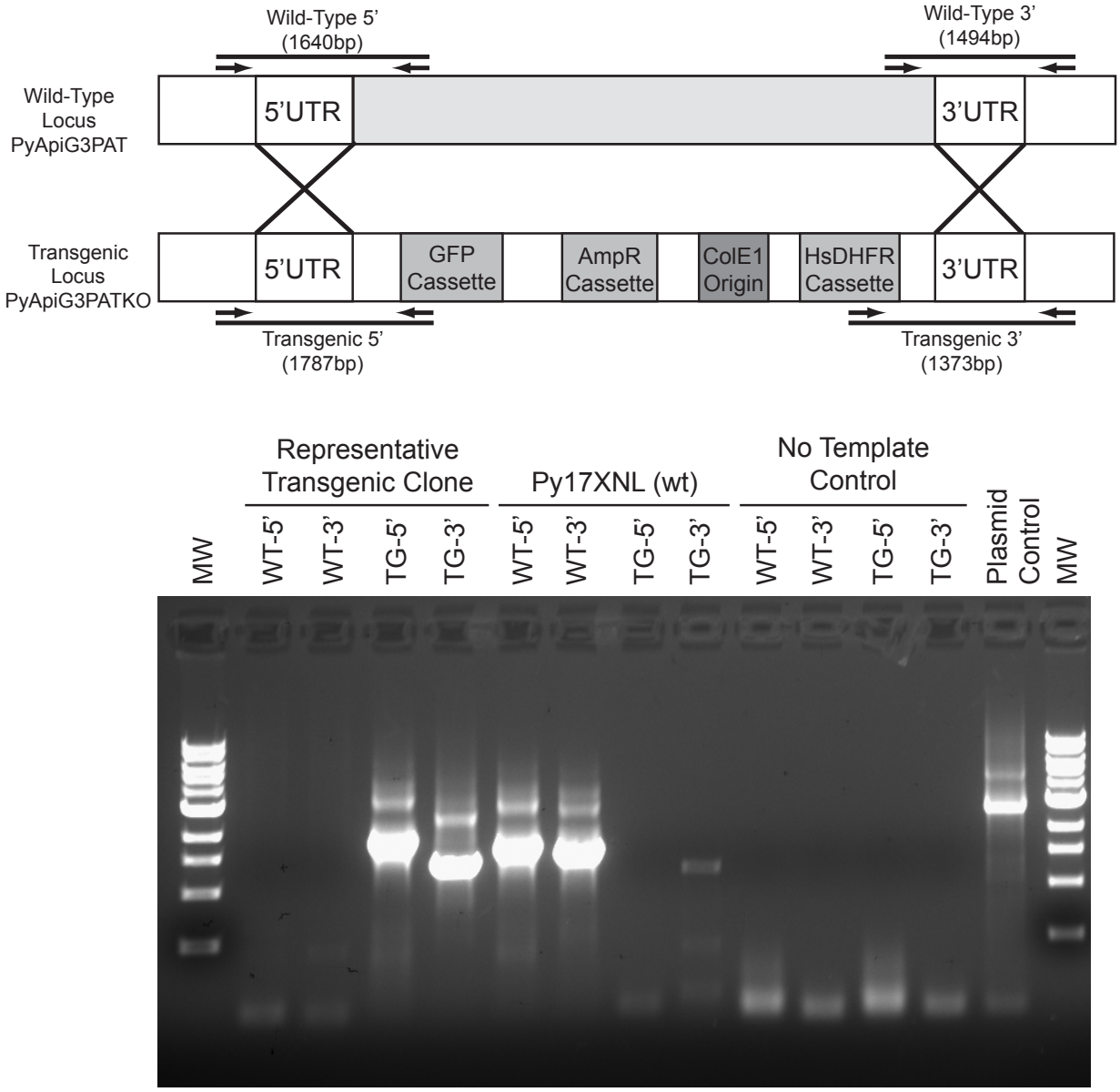
3'UTR
Transgenic
Locus
PY00789KO
5'UTR
GFP
Cassette
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(1799bp)
Transgenic 3'
(1464bp)
Representative
Transgenic Clone
Py17XNL (wt)
No Template Control
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
Plasmid
Control
MW

FIGURE S7B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(1640bp)
Wild-Type 3'
(1494bp)
Wild-Type
Locus
PyApiG3PAT
5'UTR
3'UTR
Transgenic
Locus
PyApiG3PATKO
5'UTR
GFP
Cassette
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(1787bp)
Transgenic 3'
(1373bp)
Representative
Transgenic Clone
Py17XNL (wt)
No Template
Control
MW
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
Plasmid
Control
MW

FIGURE S7C

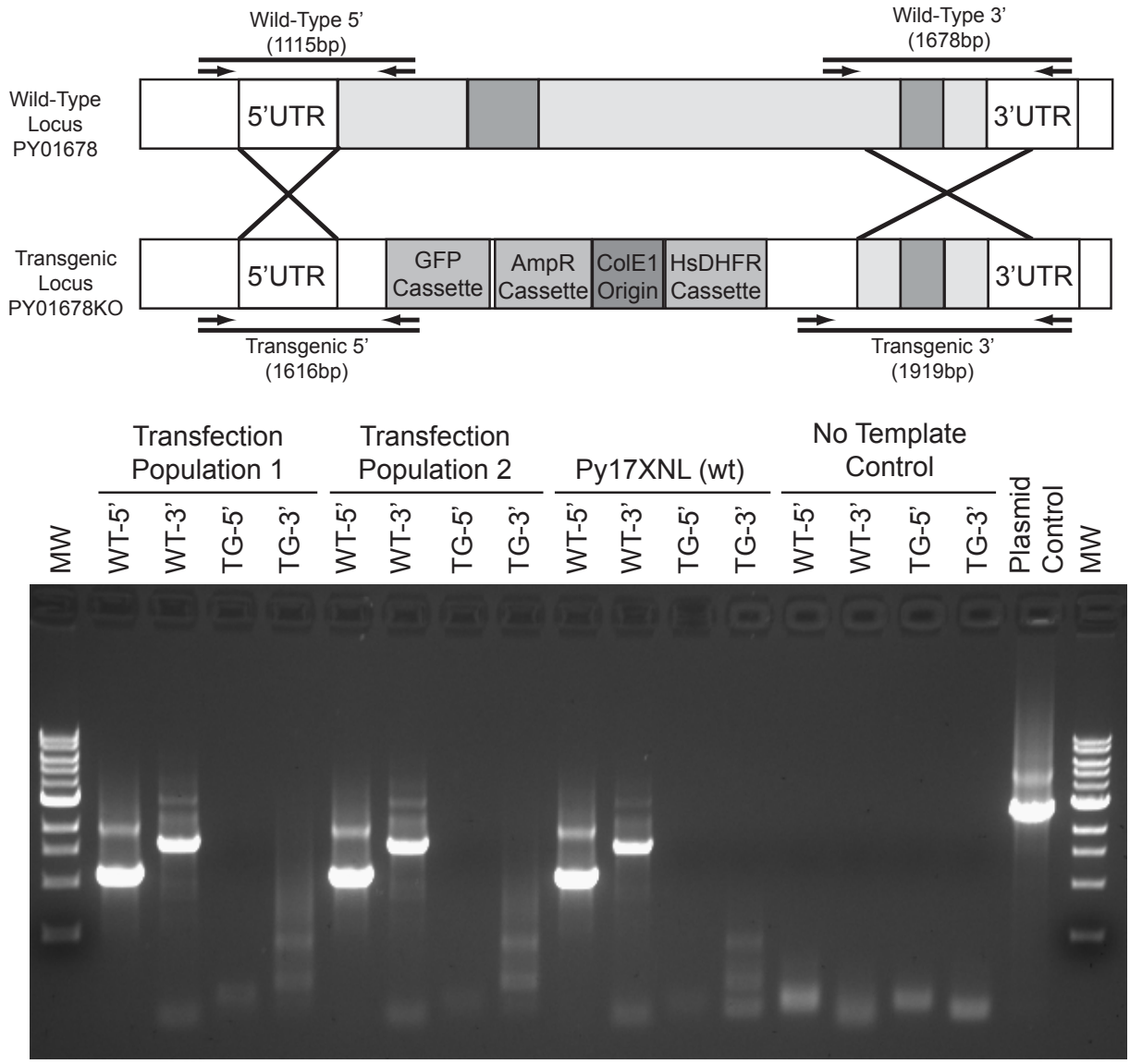

Wild-Type 5'
(1115bp)
Wild-Type 3'
(1678bp)
Wild-Type
Locus
PY01678
5'UTR
3'UTR
Transgenic
Locus
PY01678KO
5'UTR
GFP
Cassette
AmpR
Cassette
ColE1
Origin
HsDHFR
Cassette
3'UTR
Transgenic 5'
(1616bp)
Transgenic 3'
(1919bp)
Transfection
Population 1
Transfection
Population 2
Py17XNL (wt)
No Template
Control
MW
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
WT-5'
WT-3'
TG-5'
TG-3'
Plasmid
Control
MW

FIGURE S8

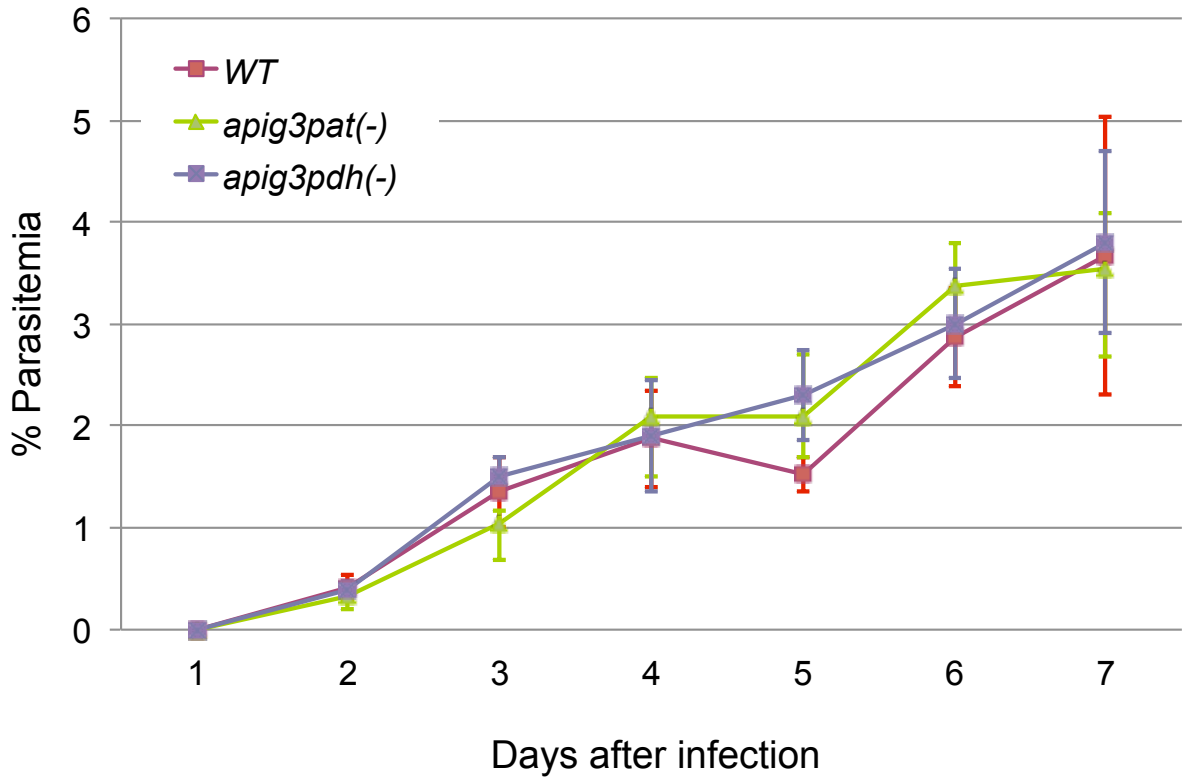
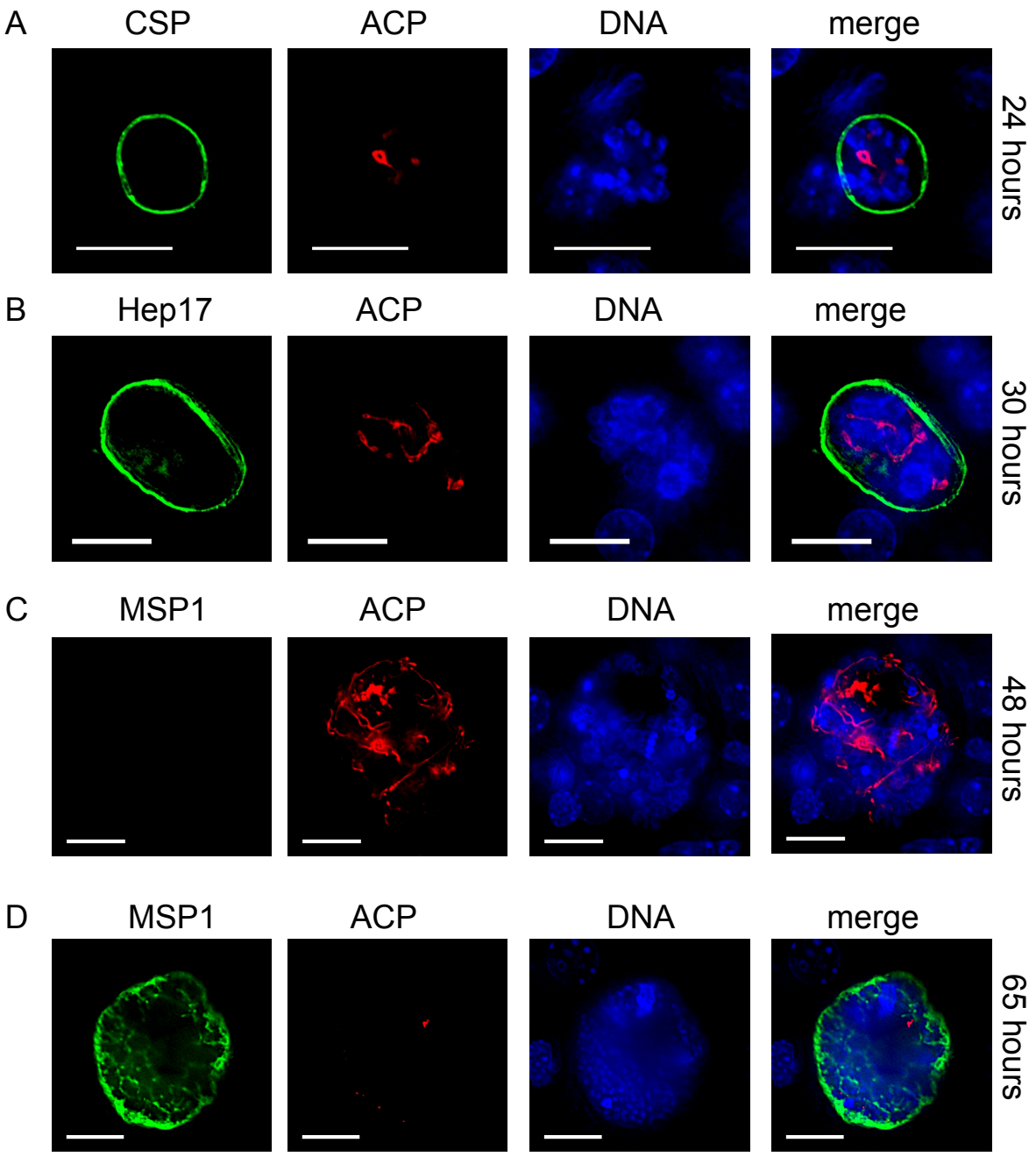

WT
apig3pat(-)
apig3pdh(-)
% Parasitemia
0
1
2
3
4
5
6
1
2
3
4
5
6
7
Days after infection

FIGURE S9


A
CSP
ACP
DNA
merge
24 hours
B
Hep17
ACP
DNA
merge
30 hours
C
MSP1
ACP
DNA
merge
48 hours
D
MSP1
ACP
DNA
merge
65 hours