Supplementary Information

Table S1. Parasitemia of single untreated *P. berghei* ANKA infected mice*. Related to Figure 1D and S1.

	Days post intravenous injection (day)						
Mouse #	5	6	7	8	9	10	14
A1	-	-	-	-	-	-	-
A2	-	-	+	+	+	+	+
A3	-	-	+	+	+	+	+
A4	-	-	-	-	-	-	-
A5	-	-	-	-	-	-	-
B1	-	-	-	-	-	-	-
B2	-	-	+	+	+	+	+
B3	-	-	-	-	-	-	-
B4	-	-	-	-	-	-	-
B5	-	-	-	-	-	-	-

* Infected Swiss Webster mouse blood was diluted and 0.5 parasite was intravenously injected to each recipient mouse. Infection was defined as blood stage parasites observed by Giemsa staining. The sensitivity of Giemsa stain is 0.01% parasitemia. Parasites were observed in mice by day 7. Recrudescence was observed in the mice at day 8-10 post ART treatment (Figure 1D and S1), implying that some parasites had entered a period of quiescence prior to reinitiating growth.

Supplementary Figures

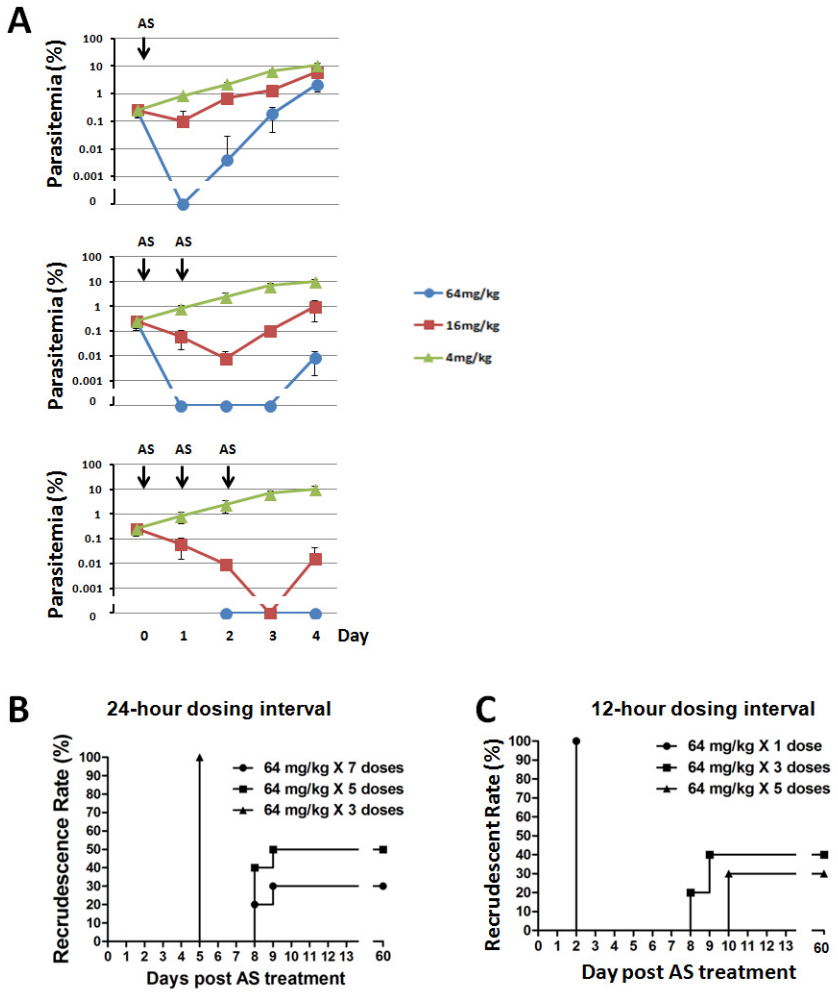

Figure S1. ***P. berghei*** **recrudescence post-AS monotherapy.** Related to Figure 1D.
(**A**) *P. berghei* ANKA infected Swiss Webster mice (5 mice / group) with parasitemia 0.2-0.4% were orally administered AS (4, 16, 64 mg/kg body weight/dose) daily for 1-3 days. Parasitemia ± SD were counted daily by Giemsa stain. (**B**) Recrudescence rate of the 64 mg/kg AS-treated mice given a 24-hour dosing interval. *P. berghei* ANKA infected mice (10 mice/group) with parasitemia 0.2-0.4% were orally administered AS (64 mg/kg body weight/dose) with a 24-hour dosing interval for 3, 5, and 7 doses, respectively. Giemsa stain was performed to detect recrudescence after the last dose. (**C**) Recrudescence rate of the 64 mg/kg AS-treated mice using a 12-hour interval. *P. berghei* ANKA infected mice (10 mice/group) with parasitemia 0.2-0.4% were orally administered AS (64 mg/kg body weight/dose) with a 12-hour dosing interval for 1, 3, and 5 doses, respectively. Giemsa staining was performed to detect recrudescence after the final dose. In mice infected with the *P. berghei* ANKA, 30-40% recrudescence occurred 8-10 days following 3-5 doses of AS at 12-hour dosing intervals. Since infection in mice was detected on day 7 following inoculation with a single untreated parasite (Table S1), the observed recrudescence at day 8-10 is due to latent parasites induced by AS. We used AS at 64 mg/kg body weight and 12-hour interval in the rest of this paper.

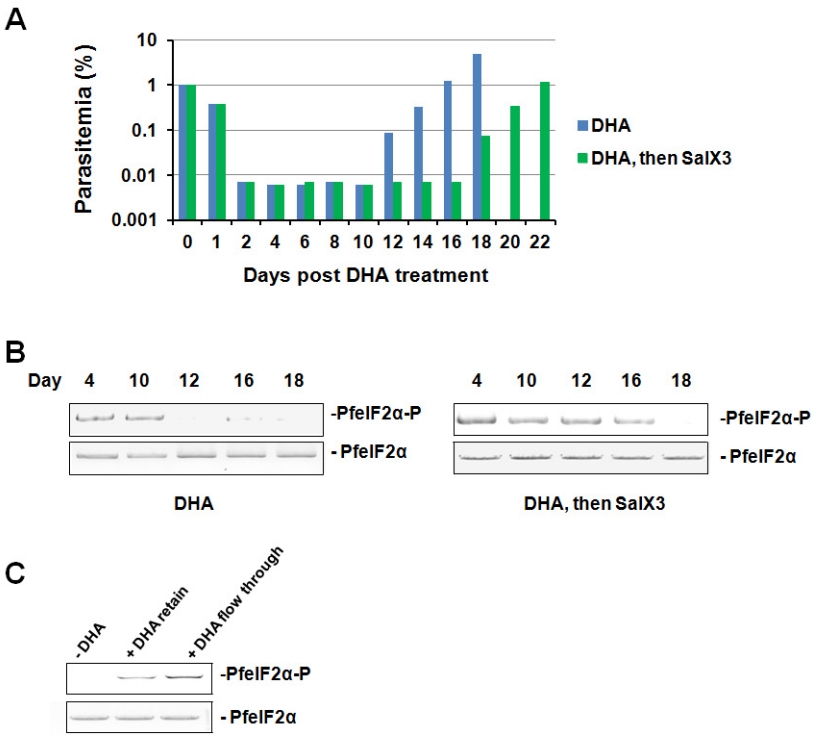

Figure S2. Sal prolongs the maintenance of PfeIF2α phosphorylation and delays the recrudescence of *P. falciparum* post DHA treatment. Related to Figure 1E.
(**A**) *P. falciparum* 3D7 cultures were treated with DHA (200 nM, 6 hours), and then incubated with 10 μM Sal at days 2, 4, and 6 post-DHA treatment. Parasites were then stained with the dormancy marker rhodamine 123, and analyzed by flow cytometry as previously described by Peatey et al. The latent parasite numbers were constant +/-Sal, and recrudescence occurred after removal of Sal, indicating that 10 μM Sal does not result in parasite death. (**B**) Sal prolonged the maintenance of elevated levels of PfeIF2α phosphorylation. The amounts of total and phosphorylated PfeIF2α were evaluated by immunoblots in parasites prepared at the specified dates post DHA treatment. (**C**) Magnetic columns were used to remove unaffected parasites by DHA treatment on day 1-3. The unaffected parasites were in replicative forms post DHA treatment, and the replicative late stage parasites were retained on magnetic column. The parasite retained on magnetic column and the latent parasites in the flow-through were subjected to immuoblots. Early stage (ring) parasites without drug treatment were included as a control. These data are representative of the results of 2 independent experiments.

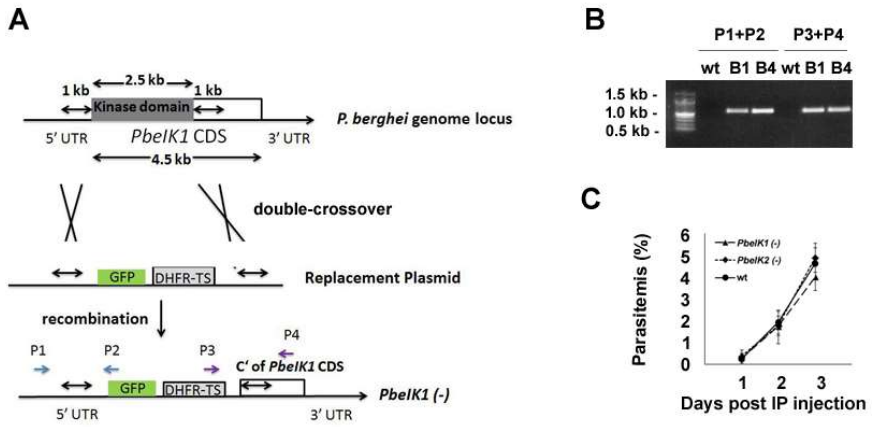

Figure S3. Generation of *PbeIK1(-)* parasites. Related to Figure 3.
(**A**) Double-crossover integration for the allelic replacement at the *PbeIK1* locus, which confers pyrimethamine resistance and results in the disruption of the *PbeIK1* gene. The replacement plasmid pBC_PbeIK1KO contains a 1-kb DNA fragment encoding the 5'-UTR of *PbeIK1*, the GFP cassette, the mutated dihydrofolate reductase (DHFR) gene that confers resistance to pyrimethamine, and a 1-kb DNA fragment encoding the *PbeIK1* CDS (position 2500bp – 3500bp). (**B**) PCR analysis for determining of allelic replacement. Confirmation of *PbeIK1* gene disruption was determined using primers P1+P2 or P3+P4, which amplify a 950-bp or a 1206-bp DNA segment from the genomic DNA of mutant clone, respectively. (**C**) *PbeIK1* (-) parasites develop normally in mouse blood. Swiss Webster mice (5 per group) were injected intraperitoneally with 10^7 *PbeIK1* (-), *PbeIK2* (-) or wt *P. berghei* ANKA parasites. The parasitemia ± SD of the recipient mice was checked in Giemsa-stained blood smears. Data are representative of 3 independent experiments.

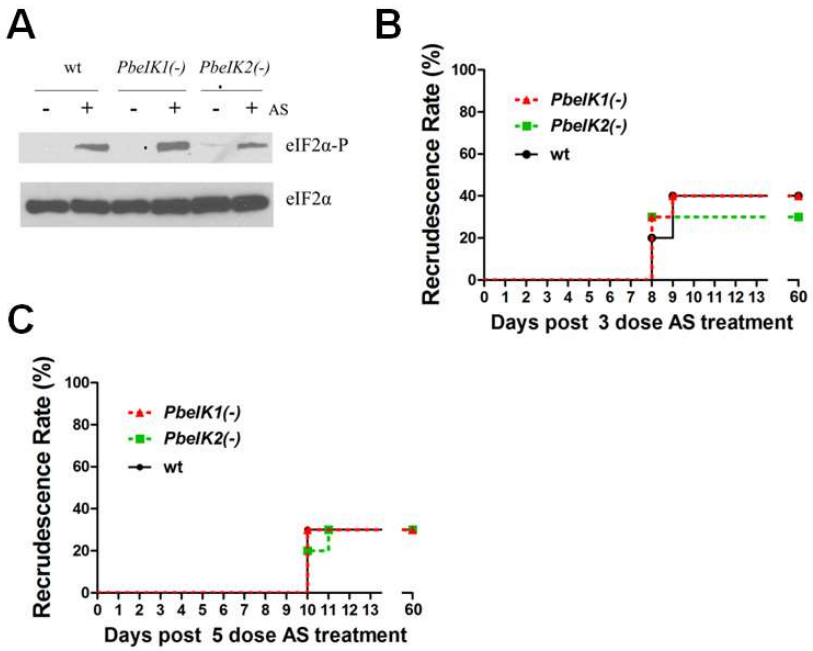

Figure S4. PbeIK1 and PbeIK2 do not respond to AS. Related to Figure 3.

(A) Immunoblot measurements of the levels of of eIF2α-P and total eIF2α in *P. berghei* ANKA before and after 3 doses of 64 mg/kg AS. (**B**) Recrudescence rate of *PbeIK1(-)* and *PbeIK2(-)* parasites after 3 doses of 64 mg/kg AS. *P. berghei* ANKA (wt), *PbeIK1(-)*, and *PbeIK2(-)* infected mice were orally administered 3 dose of 64 mg/kg AS. (**C**) Recrudescence rate of *PbeIK1(-)* and *PbeIK2(-)* parasites following 5 doses of 64 mg/kg AS. Data are representative of 3 independent experiments.

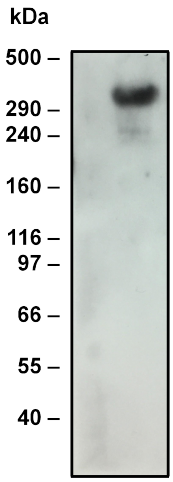

Figure S5. Mouse anti-PK4 serum. Related to Figure 3.
The GST-fused PK4 C-terminus (outside of the kinase domain) (PDYVKIHLNPWYILMLQMSKPNPADRPSAADVYSKIKVLLDPHLTDFAFSFND-IHNEHMNKPPQGTNNFERITDNKDKFVQSVVDMKNKVENEEIPIEKGLNSNVENIKNEN NGADK) was expressed in *E. coli* and the purified protein was used to immunize BALB/c mice. Immunoblot analysis of *P. berghei* parasite lysates using the prepared mouse serum (1:3,000 dilution) revealed a major band >290 kD indicative of endogenous PbPK4 (predicted 310 kDa).

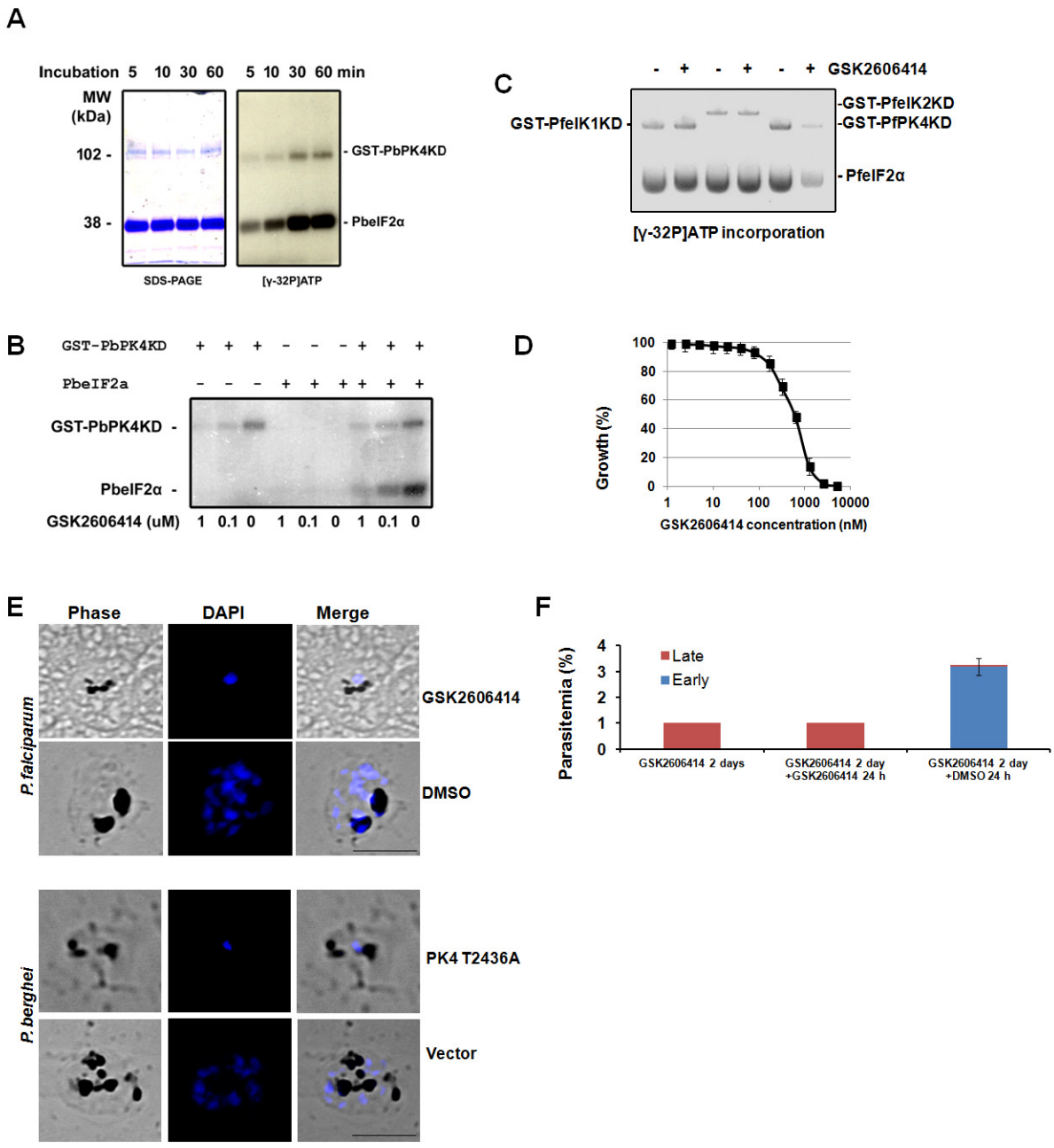

Figure S6. GSK2606414 inhibits PK4KD activity *in vitro*. Related to Figure 5.

(**A**) GST-PbPK4KD (kinase domain) and PbeIF2α were incubated with [γ-^{32}P]ATP at 37°C for 5-60 min. Proteins were separated by SDS-PAGE, followed by Coomassie blue staining and autoradiography. Note the autophosphorylation of PK4 and phosphorylation of PbeIF2α. (**B**) GSK2606414 inhibited phosphorylation of both GST-PbPK4KD and PbeIF2α *in vitro*. GST-PbPK4KD was pre-incubated with 2 μM GSK2606414 for 0.5 h, and then PbeIF2α and [γ-^{32}P]ATP were added for 30 min before resolution by SDS-PAGE and autoradiography. (**C**) The same experiment described in B was carried out except the enzyme sources were the kinase domains of each of the *P. falciparum* eIF2α kinases, PfeIK1, PfeIK2, and PfPK4. Data are representative of 3 independent experiments. (**D**) Growth inhibition of *P. falciparum* 3D7 after

incubation with GSK2606414 for 96 hours. The parasite load ± SD was evaluated by SYBR Green I nucleic acid staining dye. The EC_{50} is 0.63 μM, and the EC_{95} is 1.98 μM. (**E**) Top panel: *P. falciparum* 3D7 rings (0-3 hours post invasion) were incubated with 2 μM GSK2606414. Thirty hours later, the parasite nucleus was stained with DAPI. The parasite differentiated into schizont in absence of GSK2606414, and the differentiation into schizont was inhibited by GSK2606414. Bottom panel: The dominant-negative PK4 (T2436A mutant)-transfected schizonts were intravenously injected to a recipient mouse. Fifteen hours later, the parasite nucleus was stained with DAPI. The parasite differentiated into schizont in vector control, and the differentiation into schizont was inhibited by the dominant-negative PK4. (**F**) Parasite growth after removal of GSK2606414. Two μM of GSK2606414 was added to the ring stage of *P. falciparum* 3D7, and 2 days later the parasites stalled at late stage were refreshed with culture medium. Another 24 hours later, parasitemia ± SD was counted after Giemsa stains.