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***Plasmodium falciparum* K13 mutations in Africa and Asia impact artemisinin resistance and parasite fitness**

3

4 Barbara H. Stokes¹, Satish K. Dhingra¹, Kelly Rubiano¹, Sachel Mok¹, Judith Straimer¹, Nina F. Gnädig¹, Ioanna
5 Deni¹, Kyra A. Schindler¹, Jade R. Bath¹, Kurt E. Ward^{1,2}, Josefine Striepen¹, Tomas Yeo¹, Leila S. Ross¹, Eric
6 Legrand³, Frédéric Arieu⁴, Clark H. Cunningham^{1,5}, Issa M. Souleymane⁶, Adama Gansané⁷, Romaric
7 Nzoumbou-Boko⁸, Claudette Ndayikunda⁹, Abdunoor M. Kabanywanyi¹⁰, Aline Uwimana¹¹, Samuel J. Smith¹²,
8 Olimatou Kolley¹³, Mathieu Ndounga¹⁴, Marian Warsame¹⁵, Rithea Leang¹⁶, François Nosten^{17,18}, Timothy J.C.
9 Anderson¹⁹, Philip J. Rosenthal²⁰, Didier Ménard³, David A. Fidock^{1,21†}

10

11 ¹Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York, NY, USA;
12 ²Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand; ³ Malaria Genetics
13 and Resistance Unit, Institut Pasteur, INSERM U1201, CNRS ERL9195, Paris, France; ⁴Institut Cochin INSERM
14 U1016, Université Paris Descartes, Paris, France; ⁵Department of Genetics, University of North Carolina at
15 Chapel Hill, Chapel Hill, NC, USA; ⁶Programme National de Lutte Contre le Paludisme au Tchad, Ndjamen, Chad;
16 ⁷Centre National de Recherche et de Formation sur le Paludisme, Ouagadougou, Burkina Faso;
17 ⁸Laboratory of Parasitology, Institute Pasteur of Bangui, Bangui, Central African Republic; ⁹University Teaching
18 Hospital of Kamenge, Bujumbura, Burundi; ¹⁰Ifakara Health Institute, Dar es Salaam, Tanzania; ¹¹Malaria and
19 Other Parasitic Diseases Division, Rwanda Biomedical Centre, Kigali, Rwanda; ¹²National Malaria Control
20 Programme, Sierra Leone; ¹³National Malaria Control Program, Banjul, The Gambia; ¹⁴Programme National de
21 Lutte Contre le Paludisme, Brazzaville, République du Congo; ¹⁵School of Public Health and Community
22 Medicine, University of Gothenburg, Sweden; ¹⁶National Center for Parasitology, Entomology & Malaria Control,
23 Phnom Penh, Cambodia; ¹⁷Shoklo Malaria Research Unit, Mahidol-Oxford Tropical Medicine Research Unit,
24 Faculty of Tropical Medicine, Mahidol University, Mae Sot, Thailand; ¹⁸Centre for Tropical Medicine and Global
25 Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK; ¹⁹Texas Biomedical Research
26 Institute, San Antonio, TX, USA; ²⁰Department of Medicine, University of California, San Francisco, CA, USA;
27 ²¹Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New
28 York, NY, USA

29 ‡Corresponding author. Email: df2260@cumc.columbia.edu

30 Abstract

31 The emergence of mutant K13-mediated artemisinin (ART) resistance in *Plasmodium falciparum* malaria
32 parasites has led to widespread treatment failure across Southeast Asia. In Africa, *K13*-propeller genotyping
33 confirms the emergence of the R561H mutation in Rwanda and highlights the continuing dominance of wild-type
34 K13 elsewhere. Using gene editing, we show that R561H, along with C580Y and M579I, confer elevated *in vitro*
35 ART resistance in some African strains, contrasting with minimal changes in ART susceptibility in others. C580Y
36 and M579I cause substantial fitness costs, which may slow their dissemination in high-transmission settings, in
37 contrast with R561H that in African 3D7 parasites is fitness neutral. In Cambodia, *K13* genotyping highlights the
38 increasing spatio-temporal dominance of C580Y. Editing multiple K13 mutations into a panel of Southeast Asian
39 strains reveals that only the R561H variant yields ART resistance comparable to C580Y. In Asian Dd2 parasites
40 C580Y shows no fitness cost, in contrast with most other K13 mutations tested, including R561H. Editing point
41 mutations in *ferredoxin* or *mdr2*, earlier associated with resistance, has no impact on ART susceptibility or
42 parasite fitness. These data underline the complex interplay between K13 mutations, parasite survival, growth
43 and genetic background in contributing to the spread of ART resistance.

44 Introduction

45 Despite recent advances in chemotherapeutics, diagnostics and vector control measures, malaria continues to
46 exert a significant impact on human health (Hanboonkunupakarn and White, 2020). In 2019, cases were
47 estimated at 229 million, resulting in 409,000 fatal outcomes, primarily in Sub-Saharan Africa as a result of
48 *Plasmodium falciparum* infection (WHO, 2020). This situation is predicted to worsen as a result of the ongoing
49 SARS-CoV-2 pandemic that has compromised malaria treatment and prevention measures (Sherrard-Smith *et al.*,
50 2020). Absent an effective vaccine, malaria control and elimination strategies are critically reliant on the
51 continued clinical efficacy of first-line artemisinin-based combination therapies (ACTs) (White *et al.*, 2014).
52 These ACTs pair fast-acting artemisinin (ART) derivatives with partner drugs such as lumefantrine,
53 amodiaquine, mefloquine or piperazine (PPQ). ART derivatives can reduce the biomass of drug-sensitive
54 parasites by up to 10,000-fold within 48 h (the duration of one intra-erythrocytic developmental cycle); however,
55 these derivatives are rapidly metabolized *in vivo*. Longer-lasting albeit slower-acting partner drugs are co-
56 administered to reduce the selective pressure for ART resistance and to clear residual parasitemias (Eastman
57 and Fidock, 2009).

58
59 *P. falciparum* resistance to ART derivatives has now swept across Southeast (SE) Asia, having first emerged a
60 decade ago in western Cambodia (Dondorp *et al.*, 2009; Noedl *et al.*, 2009; Arie *et al.*, 2014; Imwong *et al.*,
61 2020). Clinically, ART resistance manifests as delayed clearance of circulating asexual blood stage parasites
62 following treatment with an ACT but does not result in treatment failure as long as the partner drug remains
63 effective. The accepted threshold for resistance is a parasite clearance half-life (the time required for the
64 peripheral blood parasite density to decrease by 50%) of >5.5 h. Sensitive parasites are typically cleared in <2-3
65 h (WHO, 2019). Resistance can also be evidenced as parasite-positive blood smears on day three post initiation
66 of treatment. *In vitro*, ART resistance manifests as an increase in survival in tightly synchronized ring-stage
67 parasites (0-3 h post invasion) exposed to a 6 h pulse of 700 nM dihydroartemisinin (DHA, the active metabolite
68 of all ARTs used clinically) in the ring-stage survival assay (RSA) (Witkowski *et al.*, 2013; Arie *et al.*, 2014).
69 Recently, ART-resistant strains have also acquired resistance to PPQ, which is widely used in SE Asia as a
70 partner drug in combination with DHA (Wicht *et al.*, 2020). Failure rates following DHA-PPQ treatment now
71 exceed 50% in parts of Cambodia, Thailand and Vietnam (van der Pluijm *et al.*, 2019).

72

73 *In vitro* selections, supported by clinical epidemiological data, have demonstrated that ART resistance is
74 primarily determined by mutations in the beta-propeller domain of the *P. falciparum* Kelch protein K13, also
75 known as Kelch13 (Ariey *et al.*, 2014; Ashley *et al.*, 2014; MalariaGEN, 2016; Menard *et al.*, 2016; Siddiqui *et*
76 *al.*, 2020). Recent evidence suggests that these mutations result in reduced endocytosis of host-derived
77 hemoglobin and thereby decrease the release of Fe²⁺-heme that serves to activate ART, thus reducing its
78 potency (Yang *et al.*, 2019; Birnbaum *et al.*, 2020). Mutations in other genes including *ferredoxin* (*fd*) and
79 *multidrug resistance protein 2* (*mdr2*) have also been associated with ART resistance in K13 mutant parasites,
80 leading to the suggestion that they either contribute to a multigenic basis of resistance or fitness or serve as
81 genetic markers of founder populations (Miotto *et al.*, 2015).

82

83 In SE Asia, the most prevalent K13 mutation is C580Y, which associates with delayed clearance *in vivo* (Ariey *et*
84 *al.*, 2014; Ashley *et al.*, 2014; MalariaGEN, 2016; Menard *et al.*, 2016; Imwong *et al.*, 2017). This mutation also
85 mediates ART resistance *in vitro*, as demonstrated by RSAs performed on gene-edited parasites (Ghorbal *et al.*,
86 2014; Straimer *et al.*, 2015; Straimer *et al.*, 2017; Mathieu *et al.*, 2020; Uwimana *et al.*, 2020). Other studies
87 have documented the emergence of nearly 200 other K13 mutations, both in SE Asia and in other malaria-
88 endemic regions, including the Guiana Shield and the western Pacific (MalariaGEN, 2016; Menard *et al.*, 2016;
89 Das *et al.*, 2019; WWARN, 2019; Mathieu *et al.*, 2020; Miotto *et al.*, 2020). Aside from C580Y, however, only a
90 handful of K13 mutations (N458Y, M476I, Y493H, R539T, I543T and R561H) have been validated by gene-
91 editing experiments as conferring resistance *in vitro* (Straimer *et al.*, 2015; Siddiqui *et al.*, 2020). Nonetheless,
92 multiple other mutations in this gene have been associated with the clinical delayed clearance phenotype and
93 have been proposed as candidate markers of ART resistance (WWARN, 2019; WHO, 2019).

94

95 Here, we define the role of a panel of K13 mutations identified in patient isolates, and address the key question
96 of whether these mutations can confer resistance in African strains. We include the K13 R561H mutation, earlier
97 associated with delayed parasite clearance in SE Asia (Ashley *et al.*, 2014; Phyo *et al.*, 2016), and very recently
98 identified at up to 13% prevalence in certain districts in Rwanda (Uwimana *et al.*, 2020; Bergmann *et al.*, 2021;
99 Uwimana *et al.*, 2021). This study also enabled us to assess the impact of the parasite genetic background on *in*
100 *vitro* phenotypes, including mutations in *ferredoxin* and *mdr2* that were earlier associated with resistance (Miotto

101 *et al.*, 2015). Our results show that K13 mutations can impart ART resistance across multiple Asian and African
102 strains, at levels that vary widely depending on the mutation and the parasite genetic background. Compared
103 with K13 mutant Asian parasites, we observed stronger *in vitro* fitness costs in most K13-edited African strains,
104 which might predict a slower dissemination of ART resistance in high-transmission African settings.
105 Nonetheless, our data highlight the threat of the R561H mutation emerging in Rwanda, which confers elevated
106 RSA resistance and minimal fitness cost in African 3D7 parasites.

107

108 Results

109 Non-synonymous K13 mutations are present at low frequencies in Africa

110 To examine the status of K13 mutations across Africa, we analyzed *K13* beta-propeller domain sequences in
111 3,257 isolates from 11 malaria-endemic African countries, including The Gambia, Sierra Leone, and Burkina
112 Faso in West Africa; Chad, Central African Republic, Republic of the Congo, and Equatorial Guinea in Central
113 Africa; and Burundi, Tanzania, Rwanda, and Somalia in East Africa. Samples were collected between 2012 and
114 2019, with most countries sampled across multiple years. 1,038 (32%) originated from The Gambia, Republic of
115 the Congo and Burundi and have not been previously reported, whereas the remaining samples including those
116 from Rwanda have been published (**Figure 1–Source data 1; Supplementary file 1**).

117

118 Of all samples, 98% (3,179) were K13 wild-type, i.e. they matched the 3D7 (African) reference sequence or
119 harbored a synonymous (non-coding) mutation. For individual countries, the percentage of K13 wild-type
120 samples ranged from 95% to 100% (**Figure 1; Figure 1–source data 1**). In total, we identified 35 unique non-
121 synonymous mutations in K13. Of these, only two have been validated as resistance mediators in the SE Asian
122 Dd2 strain: the M476I mutation initially identified from long-term ART selection studies and the R561H mutation
123 observed in SE Asia and Rwanda (Ariey *et al.*, 2014; Straimer *et al.*, 2015; Uwimana *et al.*, 2020).

124

125 Of the 35 non-synonymous mutations, only two were present in >6 samples: R561H (n=20, found only in
126 Rwanda, sampled from 2012 to 2015; (Uwimana *et al.*, 2020)), and A578S (n=10; observed in four African
127 countries across multiple years). Previously A578S was shown not to confer *in vitro* resistance in Dd2 (Menard

128 *et al.*, 2016). In the set of 927 genotyped Rwandan isolates, R561H accounted for 44% of mutant samples and
129 2% of all samples (**Figure 1 inset**).

130

131 **K13 R561H, M579I and C580Y mutations can confer *in vitro* artemisinin resistance in African parasites**

132 To test whether R561H can mediate ART resistance in African strains, we developed a CRISPR/Cas9-mediated
133 K13 editing strategy (**Supplementary file 2**) to introduce this mutation into 3D7 and F32 parasites. On the basis
134 of whole-genome sequence analysis of African isolates, 3D7 was recently shown to segregate phylogenetically
135 with parasites from Rwanda (Ariey *et al.*, 2014; Uwimana *et al.*, 2020). F32 was derived from an isolate from
136 Tanzania (Witkowski *et al.*, 2010). We also tested the C580Y mutation that predominates in SE Asia, as well as
137 the M579I mutation earlier identified in a *P. falciparum*-infected migrant worker in Equatorial Guinea who
138 displayed delayed parasite clearance following ACT treatment (Lu *et al.*, 2017). The positions of these residues
139 are highlighted in the K13 beta-propeller domain structure shown in **Supplementary file 3**. For 3D7, F32 and
140 other lines used herein, their geographic origins and genotypes at drug resistance loci are described in **Table 1**
141 and **Supplementary file 4**. All parental lines were cloned by limiting dilution prior to transfection. Edited
142 parasites were identified by PCR and Sanger sequencing, and cloned. These and other edited parasite lines
143 used herein are described in **Supplementary file 5**.

144

145 RSAs, used to measure *in vitro* ART susceptibility, revealed a wide range of mean survival values for K13
146 mutant lines. For 3D7 parasites, the highest RSA survival rates were observed with 3D7^{R561H} parasites, which
147 averaged 6.6% RSA survival. For the 3D7^{M579I} and 3D7^{C580Y} lines, mean RSA survival rates were both 4.8%, a 3
148 to 4-fold increase relative to the 3D7^{WT} line. No elevated RSA survival was seen in a 3D7 control line (3D7^{ctrl})
149 that expressed only the silent shield mutations used at the guide RNA cut site (**Figure 2A; Figure 2–source**
150 **data 1**). Western blot analysis with tightly synchronized ring-stage parasites revealed a ~30% reduction in K13
151 protein expression levels in these three K13 mutant lines relative to the parental 3D7^{WT} (**Figure 2–figure**
152 **supplement 1; Figure 2–figure supplement 1–source data 1**).

153

154 Interestingly, for F32 parasites the introduction of K13 mutations yielded no significant increase in RSA survival,
155 whose rates were in the range of 0.3% to 0.5% for lines expressing R561H, M579I, C580Y or wild-type K13.
156 (**Figure 2B**). Previously we reported that introduction of M476I into F32 parasites resulted in a modest gain of

157 resistance (mean survival of 1.7%) while this same mutation conferred RSA survival levels of ~10% in edited
158 Dd2 parasites (Straimer *et al.*, 2015). These data suggest that while K13 mutations differ substantially in their
159 impact on ART susceptibility, there is an equally notable contribution of the parasite genetic background.

160

161 We next introduced M579I and C580Y into cloned Ugandan isolates UG659 and UG815. Editing of both
162 mutations into UG659 yielded moderate RSA survival rates (means of 6.3% and 4.7% for UG659^{M579I} or
163 UG659^{C580Y} respectively, vs. 1.0% for UG659^{WT}; **Figure 2C**). These values resembled our results with 3D7.
164 Strikingly, introducing K13 M579I or C580Y into UG815 yielded the highest rates of *in vitro* resistance, with
165 mean survival levels reaching ~12% in both UG815^{M579I} and UG815^{C580Y}. These results were confirmed in a
166 second independent clone of UG815^{M579I} (**Figure 2D**). M579I and C580Y also conferred equivalent levels of
167 resistance in edited Dd2 parasites (RSA survival rates of 4.0% and 4.7%, respectively; **Figure 2—source data**
168 **1**). These data show that mutant K13-mediated ART resistance in African parasites can be achieved, in some
169 but not all strains, at levels comparable to or above those seen in SE Asian parasites.

170

171 **K13 C580Y and M579I mutations, but not R561H, are associated with an *in vitro* fitness defect across** 172 **African parasites**

173 To examine the relation between resistance and fitness in African parasites harboring K13 mutations, we
174 developed an *in vitro* fitness assay that uses quantitative real-time PCR (qPCR) for allelic discrimination. Assays
175 were conducted by pairing K13 wild-type lines (i.e. 3D7, F32, UG659 and UG815) with their isogenic edited
176 R561H, M579I, or C580Y counterparts.

177

178 Assays were initiated with tightly synchronized trophozoites, mixed in 1:1 ratios of wild-type to mutant isogenic
179 parasites, and cultures were maintained over a period of 36 days (~18 generations of asexual blood stage
180 growth). Cultures were sampled every four days for genomic DNA (gDNA) preparation and qPCR analysis.
181 TaqMan probes specific to the *K13* wild-type or mutant (R561H, M579I or C580Y) alleles were used to quantify
182 the proportion of each allele.

183

184 Results showed that the K13 M579I and C580Y mutations each conferred a significant fitness defect across
185 most strains tested, with the proportions of K13 mutant lines declining over time. For both mutations, the largest

186 reductions were observed with edited 3D7 or UG815 parasites. In contrast, these mutations exerted a minimal
187 impact on fitness in UG659. For R561H, we observed no impact on fitness in 3D7 parasites, although in F32 this
188 mutation exerted a fitness defect similar to M579I and C580Y (**Figure 3A–D; Figure 3–source data 1**). From
189 these data, we calculated the fitness cost, which represents the percent reduction in growth rate per 48 h
190 generation of a test line compared to its wild-type isogenic comparator. These costs ranged from <1% to 12%
191 per generation across mutations and lines, with the lowest costs observed in 3D7^{R561H} and UG659^{C580Y}, and the
192 greatest cost observed with *K13*-edited UG815 lines (**Figure 3E**). Comparing data across these four African
193 strains revealed that high RSA survival rates were generally accompanied by high fitness costs, and conversely
194 that low fitness costs were associated with low survival rates. An exception was 3D7^{R561H} that showed moderate
195 resistance with no apparent fitness cost (**Figure 3F**).

196

197 **The K13 C580Y mutation has swept rapidly across Cambodia, displacing other K13 variants**

198 We next examined the spatio-temporal distribution of *K13* alleles in Cambodia, the epicenter of ART resistance
199 in SE Asia. In total, we analyzed the *K13* propeller domain sequences from 3,327 parasite isolates collected
200 from western, northern, eastern and southern Cambodian provinces (**Figure 4–figure supplement 1**). Samples
201 were collected between 2001 and 2017, except for the southern region where sample collection was initiated in
202 2010. 1,412 samples (42%) were obtained and sequenced during the period 2015-2017 and have not previously
203 been published. Earlier samples were reported in (Ariey *et al.*, 2014; Menard *et al.*, 2016). In sum, 19
204 nonsynonymous polymorphisms in *K13* were identified across all regions and years. Of these, only three were
205 present in >10 samples: Y493H (n=83), R539T (n=87) and C580Y (n=1,915). Each of these mutations was
206 previously shown to confer ART resistance *in vitro* (Straimer *et al.*, 2015). Rarer mutations included A418V,
207 I543T, P553L, R561H, P574L, and D584V (**Figure 4; Figure 4–source data 1**).

208

209 This analysis revealed a significant proportion of *K13* wild-type parasites in the early 2000s, particularly in
210 northern and eastern Cambodia, where 96% of isolates in 2001-2002 were wild type (**Figure 4**). In western
211 Cambodia, where ART resistance first emerged (Dondorp *et al.*, 2009; Noedl *et al.*, 2009), the wild-type allele
212 percentage in 2001-2002 had already fallen to 56%. This is striking given that delayed parasite clearance
213 following ACT or artesunate treatment was first documented in 2008-2009 (Noedl *et al.*, 2008; Noedl *et al.*,
214 2009).

215

216 In all four regions, the frequency of the wild-type allele declined substantially over time and the diversity of
217 mutant alleles contracted, with nearly all wild-type and non-K13 C580Y mutant parasites being replaced by
218 parasites harboring the C580Y mutation (**Figure 4**). This effect was particularly pronounced in the west and the
219 south, where the prevalence of C580Y in 2016-17 effectively attained 100%, increasing from 22% and 58%
220 respectively in the initial sample sets (**Figure 4A, D**). In northern and eastern Cambodia, C580Y also
221 outcompeted all other mutant alleles; however, 19-25% of parasites remained K13 wild type in 2016-17 (**Figure**
222 **4B, C**). These data show rapid dissemination of K13 C580Y across Cambodia.

223

224 **SE Asian K13 mutations associated with delayed parasite clearance differ substantially in their ability to**
225 **confer artemisinin resistance *in vitro***

226 Given that most K13 polymorphisms present in the field have yet to be characterized *in vitro*, we selected a set
227 of mutations to test by gene editing, namely E252Q, F446I, P553L, R561H and P574L (highlighted in
228 **Supplementary file 3**). F446I is the predominant mutation in Myanmar (Imwong *et al.*, 2020). P553L, R561H
229 and P574L have each been shown to have multiple independent origins throughout SE Asia (Menard *et al.*,
230 2016), and were identified at low frequencies in our sequencing study in Cambodia (**Figure 4**). Lastly, the
231 E252Q mutation was formerly prevalent on the Thai-Myanmar border, and, despite its occurrence upstream of
232 the beta-propeller domain, has been associated with delayed parasite clearance *in vivo* (Anderson *et al.*, 2017;
233 Cerqueira *et al.*, 2017; WWARN, 2019).

234

235 Zinc-finger nuclease- or CRISPR/Cas9-based gene-edited lines expressing K13 E252Q, F446I, P553L, R561H
236 or P574L were generated in Dd2 or Cam3.II lines expressing wild-type K13 (Dd2^{WT} or Cam3.II^{WT}) and
237 recombinant parasites were cloned. Early ring-stage parasites were then assayed for their ART susceptibility
238 using the RSA. For comparison, we included published Dd2 and Cam3.II lines expressing either K13 C580Y
239 (Dd2^{C580Y} and Cam3.II^{C580Y}) or R539T (Dd2^{R539T} and the original parental line Cam3.II^{R539T}) (Straimer *et al.*,
240 2015), as well as control lines expressing only the guide-specific silent shield mutations (Dd2^{ctrl} and Cam3.II^{ctrl}).

241

242 Both the P553L and R561H mutations yielded mean RSA survival rates comparable to C580Y (4.6% or 4.3%
243 RSA survival for Dd2^{P553L} or Dd2^{R561H}, respectively, vs 4.7% for Dd2^{C580Y}; **Figure 5A; Figure 5–source data 1**).

244 F446I and P574L showed only modest increases in survival relative to the wild-type parental line (2.0% and
245 2.1% for Dd2^{F446I} and Dd2^{P574L}, respectively, vs 0.6% for Dd2^{WT}). No change in RSA survival relative to Dd2^{WT}
246 was observed for the Dd2^{E252Q} line. The resistant benchmark Dd2^{R539T} showed a mean RSA survival level of
247 20.0%, consistent with earlier reports of this mutation conferring high-grade ART resistance *in vitro* (Straimer *et al.*, 2015; Straimer *et al.*, 2017).

249

250 In contrast to Dd2, editing of the F446I, P553L and P574L mutations into Cambodian Cam3.II^{WT} parasites did
251 not result in a statistically significant increase in survival relative to the K13 wild-type line, in part because the
252 background survival rate of Cam3.II^{WT} was higher than for Dd2^{WT}. All survival values were <3%, contrasting with
253 the Cam3.II^{R539T} parental strain that expresses the R539T mutation (~20% mean survival; **Figure 5B; Figure 5–**
254 **source data 1**). The E252Q mutation did not result in elevated RSA survival in the Cam3.II background, a result
255 also observed with Dd2. Nonetheless, ART resistance was apparent upon introducing the R561H mutation into
256 Cam3.II^{WT} parasites, whose mean survival rates exceeded the Cam3.II^{C580Y} line (13.2% vs 10.0%, respectively).
257 No elevated survival was seen in the Cam3.II^{ctrl} line expressing only the silent shield mutations used at the guide
258 RNA cut site.

259

260 **SE Asian K13 mutations do not impart a significant fitness impact on Dd2 parasites**

261 Prior studies with isogenic gene-edited SE Asian lines have shown that certain K13 mutations can exert fitness
262 costs, as demonstrated by reduced intra-erythrocytic asexual blood stage parasite growth (Straimer *et al.*, 2017;
263 Nair *et al.*, 2018). To determine the fitness impact of the K13 mutations described above, we utilized an eGFP-
264 based parasite competitive growth assay (Ross *et al.*, 2018). Dd2^{E252Q}, Dd2^{F446I}, Dd2^{P553L}, Dd2^{R561H} or Dd2^{P574L}
265 were co-cultured with an isogenic K13 wild-type eGFP⁺ Dd2 reporter line at a starting ratio of 1:1. The proportion
266 of eGFP⁺ parasites was then assessed every two days. As controls, we included Dd2^{WT}, Dd2^{bsm} and Dd2^{C580Y}.
267 These data provided evidence of a minimal impact with the F446I, P553L and C580Y mutations, with E252Q,
268 R561H and P574L having greater fitness costs when compared to Dd2^{WT} (**Figure 5C; Figure 5–figure**
269 **supplement 1; Figure 5–source data 2**). Both C580Y and P553L displayed elevated RSA survival and minimal
270 fitness cost in the Dd2 strain, providing optimal traits for dissemination (**Figure 5D**). We note that all fitness
271 costs in Dd2 were considerably lower than those observed in our four African strains (**Figure 3**).

272

273 **Strain-dependent genetic background differences significantly impact RSA survival rates in culture-**
274 **adapted Thai isolates**

275 Given the earlier abundance of the R561H and E252Q alleles in border regions of Thailand and Myanmar, we
276 next tested the impact of introducing these mutations into five Thai K13 wild-type isolates (Thai1-5). For
277 comparison, we also edited C580Y into several isolates. These studies revealed a major contribution of the
278 parasite genetic background in dictating the level of mutant K13-mediated ART resistance, as exemplified by the
279 C580Y lines whose mean survival rates ranged from 2.1% to 15.4%. Trends observed for individual mutations
280 were maintained across strains, with the R561H mutation consistently yielding moderate to high *in vitro*
281 resistance at or above the level of C580Y. Consistent with our Dd2 results, introducing E252Q did not result in
282 significant increases in survival rates relative to isogenic K13 wild-type lines (**Figure 6A-E; Figure 6–source**
283 **data 1**).

284

285 We also profiled two unedited culture-adapted Thai isolates (Thai6^{E252Q} and Thai7^{E252Q}) that express the K13
286 E252Q mutation upstream of the propeller domain. Notably, both lines exhibited mean RSA survival rates
287 significantly above the 1% threshold for ART sensitivity (2.7% for Thai6^{E252Q} and 5.1% for Thai7^{E252Q}; **Figure**
288 **6F**). These data suggest that additional genetic factors present in these two Thai isolates are required for
289 E252Q to manifest ART resistance.

290

291 **Mutations in the *P. falciparum* multidrug resistance protein 2 and ferredoxin genes do not modulate**
292 **resistance to artemisinin or parasite fitness *in vitro***

293 In a prior genome-wide association study of SE Asian parasites, K13-mediated ART resistance was associated
294 with D193Y and T484I mutations in the *ferredoxin* (*fd*) and *multidrug resistance protein 2* (*mdr2*) genes,
295 respectively (Miotto *et al.*, 2015). To directly test their role, we applied CRISPR/Cas9 editing (**Supplementary**
296 **file 6**) to revert the *fd* D193Y and *mdr2* T484I mutations to the wild-type sequences in the Cambodian strains
297 RF7^{C580Y} and Cam3.II^{C580Y}, which both express K13 C580Y. Isogenic RF7^{C580Y} parasites expressing the mutant
298 or wild-type *fd* residue at position 193 showed no change in RSA survival rates, either at 700 nM (averaging
299 ~27%), or across a range of DHA concentrations down to 1.4 nM (**Figure 7A, C; Figure 7–figure supplement**
300 **1; Figure 7–source data 1**). Editing *fd* D193Y into the recombinant CamWT^{C580Y} line that expresses K13
301 C580Y (Straimer *et al.*, 2015) also had no impact on RSA survival (with mean RSA survival rates of 11-13%).

302 Likewise, Cam3.II^{C580Y} parasites maintained the same rate of *in vitro* RSA survival (mean 19-22%) irrespective
303 of their *mdr2* allele. Silent shield mutations had no impact for either *fd* or *mdr2*. eGFP-based fitness assays
304 initiated at different starting ratios of eGFP and either *fd*-edited RF7^{C580Y} or *mdr2*-edited Cam3.II^{C580Y} lines
305 revealed no change in the growth rates of the *fd* or *mdr2* mutants compared with their wild-type controls (**Figure**
306 **7B, D; Figure 7–figure supplement 1; Figure 7–source data 2 and 3**). These data suggest that the *fd* D193Y
307 and *mdr2* T484I mutations are markers of ART-resistant founder populations but themselves do not contribute
308 directly to ART resistance or augment parasite fitness.

309

310 Discussion

311 Mutant K13-mediated ART resistance has substantially compromised the efficacy of antimalarial treatments
312 across SE Asia (Hanboonkunupakarn and White, 2020), and the relatively high prevalence of the R561H variant
313 that has recently been associated with delayed clearance in Rwanda highlights the risk of ART resistance
314 emerging and spreading in sub-Saharan Africa (Uwimana *et al.*, 2020; Bergmann *et al.*, 2021; Uwimana *et al.*,
315 2021). Using gene editing and phenotypic analyses, we provide definitive evidence that the K13 R561H, M579I
316 and C580Y mutations can confer *in vitro* ART resistance in several African strains. *In vitro* resistance, as defined
317 using the RSA, was comparable between gene-edited K13 R561H 3D7 parasites (originating from or near
318 Rwanda) and C580Y Dd2 and Cam3.II parasites (from SE Asia). Further investigations into edited African 3D7
319 parasites showed that these mutations also resulted in a ~30% decrease in K13 protein levels, consistent with
320 earlier studies into the mechanistic basis of mutant K13-mediated ART resistance (Birnbaum *et al.*, 2017;
321 Siddiqui *et al.*, 2017; Yang *et al.*, 2019; Gnadig *et al.*, 2020; Mok *et al.*, 2021). We also observed that K13
322 mutant African strains differed widely in their RSA survival rates. As an example, when introduced into the
323 Tanzanian F32 strain, the C580Y mutation yielded a 0.3% RSA survival rate (not resistant), contrasting with
324 11.8% survival (highly resistant) in the Ugandan UG815 strain. These data suggest that F32 parasites lack
325 additional genetic determinants that are required for mutant K13 to confer ART resistance. Collectively, our
326 results provide evidence that certain African strains present no major biological obstacle to becoming ART
327 resistant *in vitro* upon acquiring K13 mutations. Further gene editing experiments are merited to extend these
328 studies to additional African strains, and to incorporate other variants such as C469Y and A675V that are
329 increasing in prevalence in Uganda (Asua *et al.*, 2020).

330

331 Our mixed culture competition assays with African parasites revealed substantial fitness costs with the K13
332 C580Y mutation in three of the four strains tested (UG659 was the exception). The largest growth defect was
333 observed with the edited UG815 C580Y line, which also yielded the highest level of ART resistance *in vitro*.
334 These data suggest that K13 C580Y may not easily take hold in Africa where, unlike in SE Asia, infections are
335 often highly polyclonal, generating intra-host competition that impacts a strain's ability to succeed at the
336 population level. In addition, individuals in highly-endemic African settings generally have high levels of acquired
337 immunity, potentially minimizing infection by relatively unfit parasites, and often have asymptomatic infections
338 that go untreated, and are thus less subject to selective drug pressure, compared with individuals in SE Asia
339 (Eastman and Fidock, 2009). This situation recalls the history of chloroquine use in Africa, where fitness costs
340 caused by mutations in the primary resistance determinant PfCRT resulted in the rapid resurgence of wild-type
341 parasites following the implementation of other first-line antimalarial therapies (Kublin *et al.*, 2003; Laufer *et al.*,
342 2006; Ord *et al.*, 2007; Frosch *et al.*, 2014).

343

344 An even greater fitness cost was observed with the M579I mutation, earlier detected in an infection acquired in
345 Equatorial Guinea with evidence of *in vivo* ART resistance (Lu *et al.*, 2017) but which was notably absent in all
346 3,257 African samples reported herein. In contrast, we observed no evident fitness cost in 3D7 parasites
347 expressing the R561H variant, which might help contribute to its increasing prevalence in Rwanda. While our
348 Rwandan samples from 2012-2015 observed this mutation at 2% prevalence, samples collected by others in
349 2018 and 2019 identified this mutation at 12-13% prevalence (Bergmann *et al.*, 2021; Uwimana *et al.*, 2021).
350 One of these reports included evidence associating R561H with delayed parasite clearance in patients treated
351 with the ACT artemether-lumefantrine (Uwimana *et al.*, 2021). These recent data heighten the concern that
352 mutant K13 might be taking hold in certain areas in Africa where it can begin to compromise ACT efficacy.

353

354 In Cambodia, our spatio-temporal analysis of K13 sequence diversity highlights the initial emergence of C580Y
355 in the western provinces, and its progressive replacement of other variants in the country. Interestingly, this
356 mutation was already at high prevalence in western Cambodia several years before the first published reports of
357 delayed parasite clearance in ART-treated patients (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Arieu *et al.*, 2014).
358 The success of this mutation in Cambodia, and elsewhere in the eastern Greater Mekong subregion (Imwong *et*

359 *al.*, 2020), cannot be explained by resistance alone, as we previously reported that the less common R539T and
360 I543T variants conferred greater ART resistance *in vitro* (Straimer *et al.*, 2015). Similarly, we now report that the
361 R561H and P553L mutations yield equivalent degrees of ART resistance in Dd2 parasites when compared with
362 C580Y. In contrast, low-level resistance was observed with F446I, which has nonetheless spread across
363 Myanmar (Imwong *et al.*, 2020). In a separate recent gene editing study, F446I yielded no significant *in vitro*
364 resistance in 3D7 parasites and was fitness neutral (Siddiqui *et al.*, 2020), consistent with our findings for this
365 mutation in edited Dd2 parasites.

366

367 Our studies into the impact of K13 mutations on *in vitro* growth in Asian Dd2 parasites provide evidence that that
368 the C580Y mutation generally exerts less of a fitness cost relative to other K13 variants, as measured in K13-
369 edited parasites co-cultured with an eGFP reporter line. A notable exception was P553L, which compared with
370 C580Y was similarly fitness neutral and showed similar RSA values. P553L has nonetheless proven far less
371 successful in its regional dissemination compared with C580Y (Menard *et al.*, 2016). These data suggest that
372 additional factors have contributed to the success of C580Y in sweeping across SE Asia. These might include
373 specific genetic backgrounds that have favored the dissemination of C580Y parasites, possibly resulting in
374 enhanced transmission potential (Witmer *et al.*, 2020), or ACT use that favored the selection of partner drug
375 resistance in these parasite backgrounds (van der Pluijm *et al.*, 2019). In terms of growth rates in our isogenic
376 Dd2 lines, the most detrimental impacts were observed with E252Q and R561H, which earlier predominated
377 near the Thailand-Myanmar border region, but were later overtaken by C580Y (Phyo *et al.*, 2016). In our study,
378 C580Y produced an optimal combination of no measurable fitness cost and relatively high RSA survival rates in
379 Dd2 parasites. In a prior independent study however, R561H showed slightly improved fitness relative to C580Y
380 in paired isogenic parasites from Thailand (generated in the NHP4302 strain), providing further evidence that
381 both fitness and resistance are strain-dependent (Nair *et al.*, 2018).

382

383 Further research is also required to define secondary genetic determinants that could augment mutant K13-
384 mediated ART resistance, and to explore other potential mediators of resistance. Proposed candidates have
385 included *fd*, *mdr2*, *ap-2μ*, *ubp1* and *pfcoronin*, which have earlier been associated with *P. falciparum* ART
386 susceptibility (Demas *et al.*, 2018; Henrici *et al.*, 2019; Sutherland *et al.*, 2020). Our data argue against a direct
387 role for mutations in *fd* and *mdr2* in the strains tested herein. We also observed no evident association between

388 the genotypes of *pfprt*, *pfmdr1*, *arps10*, *ap-2μ* or *ubp1* and the degree to which mutant K13 conferred ART
389 resistance *in vitro* in our set of African or Asian strains (**Supplementary file 4**). Mutations associated with
390 enhanced DNA repair mechanisms have also been observed in ART-resistant SE Asian parasites, supporting
391 the idea that mutant K13 parasites may have an improved ability to repair ART-mediated DNA damage (Xiong
392 *et al.*, 2020). Further studies are merited to investigate whether these DNA repair mutations may provide a
393 favorable background for the development of ART resistance.

394

395 At the population level, we note that *P. falciparum* genomic structures in Africa tend to be far more diverse than
396 in the epicenter of resistance in Cambodia, where parasite strains are highly sub-structured into a few lineages
397 that can readily maintain complex genetic traits (Amato *et al.*, 2018). A requirement to transmit mutant K13 and
398 additional determinants of resistance in African malaria-endemic settings, where genetic outcrossing is the
399 norm, would predict that ART resistance will spread more gradually in this continent than in SE Asia. It is
400 nonetheless possible that secondary determinants will allow some African strains to offset fitness costs
401 associated with mutant K13, or otherwise augment K13-mediated ART resistance. Identifying such determinants
402 could be possible using genome-wide association studies or genetic crosses between ART-resistant and
403 sensitive African parasites in the human liver-chimeric mouse model of *P. falciparum* infection (Vaughan *et al.*,
404 2015; Amambua-Ngwa *et al.*, 2019). Reduced transmission rates in areas of Africa where malaria is declining,
405 leading to lower levels of immunity, may also benefit the emergence and dissemination of mutant K13 (Conrad
406 and Rosenthal, 2019).

407

408 Another impediment to the dissemination of ART resistance in Africa is the continued potent activity of
409 lumefantrine, the partner drug in the first line treatment artemether-lumefantrine (Conrad and Rosenthal, 2019).
410 This situation contrasts with SE Asia where ART-resistant parasites have also developed high-level resistance
411 to the partner drug PPQ, with widespread treatment failures enabling the dissemination of multidrug-resistant
412 strains (Conrad and Rosenthal, 2019; van der Pluijm *et al.*, 2019). While the genotyping data presented herein
413 and other recent molecular surveillance studies reveal low prevalence of mutant K13 in Africa (Kayiba *et al.*,
414 2020; Schmedes *et al.*, 2021), the emergence and spread of the R561H variant in Rwanda is cause for
415 significant concern. These data call for continuous continent-wide monitoring of the emergence and spread of

416 mutant K13 in Africa, and for studies into whether its emergence in Rwanda is a harbinger of subsequent
417 partner drug resistance and ACT treatment failure.

418 **Materials and Methods**

419 **Key Resources Table**

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Gene (<i>Plasmodium falciparum</i> 3D7 strain)	<i>Kelch13 (K13)</i>	PlasmoDB	PF3D7_1343700	
Gene (<i>Plasmodium falciparum</i> 3D7 strain)	<i>Ferredoxin (fd)</i>	PlasmoDB	PF3D7_1318100	
Gene (<i>Plasmodium falciparum</i> 3D7 strain)	<i>Multidrug resistance protein 2 (mdr2)</i>	PlasmoDB	PF3D7_1447900	
Strain, strain background (<i>Plasmodium falciparum</i>)	3D7 clone A10 (3D7 ^{WT})	D. Goldberg, Washington University School of Medicine, St. Louis, MO, USA		see Table 1 and Supplementary file 4 for additional details on all <i>P. falciparum</i> strains employed herein
Strain, strain background (<i>Plasmodium falciparum</i>)	F32-TEM (F32 ^{WT})	F. Benoit-Vical, Université de Toulouse, Toulouse, France (Ariey <i>et al.</i> , 2014)		
Strain, strain background (<i>Plasmodium falciparum</i>)	UG659 (UG659 ^{WT})	P. Rosenthal, University of California, San Francisco, CA, USA		
Strain, strain background (<i>Plasmodium falciparum</i>)	UG815 (UG815 ^{WT})	P. Rosenthal, University of California, San Francisco, CA, USA		
Strain, strain background (<i>Plasmodium falciparum</i>)	Dd2 (Dd2 ^{WT})	The Malaria Research and Reference Reagent Resource Center (MR4), BEI Resources	MRA-156	
Strain, strain background (<i>Plasmodium falciparum</i>)	Cam3.II (Cam3.II ^{R539T})	R. Fairhurst, NIAID, NIH, Bethesda, MD, USA (Straimer <i>et al.</i> , 2015)	PH0306-C	

Strain, strain background (<i>Plasmodium falciparum</i>)	CamWT	R. Fairhurst, NIAID, NIH, Bethesda, MD, USA (Straimer <i>et al.</i> , 2015)	PH0164-C	
Strain, strain background (<i>Plasmodium falciparum</i>)	RF7 (RF7 ^{C580Y})	R. Fairhurst, NIAID, NIH, Bethesda, MD, USA (Ross <i>et al.</i> , 2018)	PH1008-C	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai1 ^{WT}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	TA32A2A4	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai2 ^{WT}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	TA50A2B2	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai3 ^{WT}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	TA85R1	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai4 ^{WT}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	TA86A3	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai5 ^{WT}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	NHP-01334-6B	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai6 ^{E252Q}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	NHP4076	
Strain, strain background (<i>Plasmodium falciparum</i>)	Thai7 ^{E252Q}	T. Anderson, Texas Biomedical Research Institute, San Antonio, TX, USA	NHP4673	
Strain, strain background (<i>Escherichia coli</i>)	HST08	Takara	Cat. #636766	Stellar™ Competent Cells

Genetic reagent (<i>Plasmodium falciparum</i>)	Transgenic parasite lines	This study and (Straimer <i>et al.</i> , 2015)	See Supplementary file 5	Available from D. Fidock upon request
Commercial assay or kit	In-Fusion® HD Cloning Plus kit	Takara	Cat. #638909	
Commercial assay or kit	QuantiFast Multiplex PCR Kit	Qiagen	Cat. #204654	
Sequence-based reagents	Oligonucleotides	This study	See Supplementary file 7	
Recombinant DNA reagents	Plasmids	This study	See Supplementary file 8	Available from D. Fidock upon request
Sequence-based reagents	qPCR primers and probes	This study	See Supplementary file 9	
Antibody	Anti-K13 (<i>P. falciparum</i>) (Mouse monoclonal)	I. Trakht, Columbia University Medical Center, New York, NY, USA (Gnadig <i>et al.</i> , 2020)		Antibody clone E9 WB (1:1000)
Antibody	Anti-ERD2 (<i>P. falciparum</i>) (Rabbit polyclonal)	MR4, BEI Resources	MRA-1	WB (1:1000)
Antibody	StarBright Blue 700 goat anti-mouse	Bio-Rad	12004158	WB (1:200)
Antibody	StarBright Blue 520 goat anti-rabbit	Bio-Rad	12005869	WB (1:1000)
Other	4–20% Criterion™ TGX™ Precast Protein Gel	Bio-Rad	5671093	Used with recommended buffers, also purchased from Bio-Rad
Chemical compound, drug	Carbenicillin disodium salt	Sigma	C1389	
Chemical compound, drug	WR99210	Jacobus Pharmaceuticals		
Chemical compound, drug	Dihydroartemisinin (DHA)	Sigma	D7439	

Software, algorithm	GraphPad Prism Version 9	GraphPad Software, San Diego, CA, USA		graphpad.com
Software, algorithm	ImageJ software	NIH, Bethesda, MD, USA		imagej.nih.gov

Sample collection and *K13* genotyping

Samples were obtained as blood-spot filter papers from patients seeking treatment at sites involved in national surveys of antimalarial drug resistance, or patients enrolled in therapeutic efficacy studies, or asymptomatic participants enrolled in surveillance programs. Collection details from African and Cambodian samples are provided in **Figure 1–source data 1** and **Figure 4–source data 1**, respectively. Samples were processed at the Pasteur Institute in Paris or the Pasteur Institute in Cambodia, as detailed in **Supplementary file 1**. These investigators vouch for the accuracy and completeness of the molecular data. DNA was extracted from dried blood spots using QIAmp Mini kits, as described (Menard *et al.*, 2016). A nested PCR was performed on each sample to amplify the *K13*-propeller domain, corresponding to codons 440-680. PCR products were sequenced using internal primers and electropherograms analyzed on both strands, using the Pf3D7_1343700 3D7 sequence as the wild-type reference. Quality controls included adding six blinded quality-control samples to each 96-well sequencing plate prepared from samples from each in-country partner and independently retesting randomly selected blood samples. Isolates with mixed alleles were considered to be mutated for the purposes of estimating the mutation frequencies.

P. falciparum parasite *in vitro* culture

Plasmodium falciparum asexual blood-stage parasites were cultured in human erythrocytes at 3% hematocrit in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mM HEPES, 0.21% NaHCO₃, 10 mg/L gentamycin and 0.5% w/v Albumax II (Invitrogen). Parasites were maintained at 37°C in 5% O₂, 5% CO₂, and 90% N₂. The geographic origin and year of culture adaptation for lines employed herein are described in **Supplementary file 4**. Parasite lines were authenticated by genotyping resistance genes and were screened by PCR for Mycoplasma every 3-6 months.

444 Whole-genome sequencing of parental lines

445 To define the genome sequences of our *P. falciparum* lines used for transfection, we lysed parasites in 0.05%
446 saponin, washed them with 1×PBS, and purified genomic DNA (gDNA) using the QIAamp DNA Blood Midi Kit
447 (Qiagen). DNA concentrations were quantified by NanoDrop (Thermo Scientific) and Qubit (Invitrogen) prior to
448 sequencing. 200 ng of gDNA was used to prepare sequencing libraries using the Illumina Nextera DNA Flex
449 library prep kit with dual indices. Samples were multiplexed and sequenced on an Illumina MiSeq to obtain 300
450 bp paired-end reads at an average of 50× depth of coverage. Sequence reads were aligned to
451 the *P. falciparum* 3D7 reference genome (PlasmoDB version 36) using Burrow-Wheeler Alignment. PCR
452 duplicates and unmapped reads were filtered out using Samtools and Picard. Reads were realigned around
453 indels using GATK RealignerTargetCreator and base quality scores were recalibrated using GATK
454 BaseRecalibrator. GATK HaplotypeCaller (version 3.8) was used to identify all single nucleotide polymorphisms
455 (SNPs). These SNPs were filtered based on quality scores (variant quality as function of depth QD > 1.5,
456 mapping quality > 40, min base quality score > 18) and read depth (> 5) to obtain high-quality SNPs, which were
457 annotated using snpEFF. Integrated Genome Viewer was used to visually verify the presence of SNPs. BIC-Seq
458 was used to check for copy number variations using the Bayesian statistical model (Xi *et al.*, 2011). Copy
459 number variations in highly polymorphic surface antigens and multi-gene families were removed as these are
460 prone to stochastic changes during *in vitro* culture.

461
462 Whole-genome sequencing data were used to determine the genotypes of the antimalarial drug resistance loci
463 *pfcr*, *mdr1*, *dhfr* and *dhps* (Haldar *et al.*, 2018). We also genotyped *fd*, *arps10*, *mdr2*, *ubp1*, and *ap-2μ*, which
464 were previously associated with ART resistance (Henriques *et al.*, 2014; Miotto *et al.*, 2015; Cerqueira *et al.*,
465 2017; Adams *et al.*, 2018). These results are described in **Supplementary file 4**.

466

467 Cloning of *K13*, *fd* and *mdr2* plasmids

468 Zinc-finger nuclease-mediated editing of select mutations in the *K13* locus was performed as previously
469 described (Straimer *et al.*, 2015). CRISPR/Cas9 editing of *K13* mutations was achieved using the pDC2-cam-
470 coSpCas9-U6-gRNA-*hdhfr* all-in-one plasmid that contains a *P. falciparum* codon-optimized Cas9 sequence, a
471 human dihydrofolate reductase (*hdhfr*) gene expression cassette (conferring resistance to WR99210) and
472 restriction enzyme insertion sites for the guide RNA (gRNA) and donor template (White *et al.*, 2019). A *K13*

propeller domain-specific gRNA was introduced into this vector at the BbsI restriction sites using the primer pair p1+p2 (**Supplementary file 7**) using T4 DNA ligase (New England BioLabs). Oligos were phosphorylated and annealed prior to cloning. A donor template consisting of a 1.5 kb region of the *K13* coding region including the entire propeller domain was amplified using the primer pair p3+p4 and cloned into the pGEM T-easy vector system (Promega). This donor sequence was subjected to site-directed mutagenesis in the pGEM vector to introduce silent shield mutations at the Cas9 cleavage site using the primer pair p5+p6, and to introduce allele-specific mutations using primer pairs (p7 to p20). *K13* donor sequences were amplified from the pGEM vector using the primer pair p21+p22 and sub-cloned into the pDC2-cam-coSpCas9-U6-gRNA-hdfr plasmid at the EcoRI and AatII restriction sites by In-Fusion Cloning (Takara). Final plasmids (see **Supplementary file 8**) were sequenced using primers p23 to p25. A schematic showing the method of *K13* plasmid construction can be found in **Supplementary file 2**. Both our customized zinc-finger nuclease and CRISPR/Cas9 approaches generated the desired amino acid substitutions without the genomic integration of any plasmid sequences or any additional amino acid changes in the *K13* locus, and thus provide fully comparable data.

486

CRISPR/Cas9 editing of *fd* and *mdr2* was performed using a separate all-in-one plasmid, pDC2-cam-Cas9-U6-gRNA-hdfr, generated prior to the development of the codon-optimized version used above for *K13* (Lim *et al.*, 2016). Cloning was performed as for *K13*, except for gRNA cloning that was performed using In-Fusion cloning (Takara) rather than T4 ligase. Cloning of gRNAs was performed using primer pair p29+p30 for *fd* and p42+p43 for *mdr2*. Donor templates were amplified and cloned into the final vector using the primer pairs p31+p32 for *fd* and p44+p45 for *mdr2*. Site-directed mutagenesis was performed using the allele-specific primer pairs p33+p34 or p35+p36 for *fd*, and p46+p47 or p48+p49 for *mdr2*. All final plasmids (for both *fd* and *mdr2*, see **Supplementary file 8**) were sequenced using the primer pair p37+p38 (**Supplementary file 7**). Schematic representations of final plasmids are shown in **Supplementary file 6**.

496

497 **Generation of *K13*, *fd* and *mdr2* gene-edited parasite lines**

Gene-edited lines were generated by electroporating ring-stage parasites at 5-10% parasitemia with 50 µg of purified circular plasmid DNA resuspended in Cytomix. Transfected parasites were selected by culturing in the presence of WR99210 (Jacobus Pharmaceuticals) for six days post electroporation. Parental lines harboring 2-3 mutations in the *P. falciparum* dihydrofolate reductase (*dhfr*) gene were exposed to 2.5 nM WR99210, while

502 parasites harboring four *dhfr* mutations were selected under 10 nM WR99210 (see **Supplementary file 4** for
503 *dhfr* genotypes of transfected lines). Parasite cultures were monitored for recrudescence by microscopy for up
504 six weeks post electroporation. To test for successful editing, the *K13* locus was amplified directly from
505 parasitized whole blood using the primer pair p26+p27 (**Supplementary file 7**) and the MyTaq™ Blood-PCR Kit
506 (Bioline). Primer pairs p39+p40 and p50+p51 were used to amplify *fd* and *mdr2*, respectively. PCR products
507 were submitted for Sanger sequencing using the PCR primers as well as primer p28 in the case of *K13*, p41 (*fd*)
508 or p52 (*mdr2*). Bulk-transfected cultures showing evidence of editing by Sanger sequencing were cloned by
509 limiting dilution. All gene-edited transgenic lines generated herein are described in **Supplementary file 5**.

510

511 **Parasite synchronization, ring-stage survival assays (RSAs) and flow cytometry**

512 Synchronized parasite cultures were obtained by exposing predominantly ring-stage cultures to 5% D-Sorbitol
513 (Sigma) for 15 min at 37°C to remove mature parasites. After 36 h of subsequent culture, multinucleated
514 schizonts were purified over a density gradient consisting of 75% Percoll (Sigma). Purified schizonts were
515 incubated with fresh RBCs for 3h, and early rings (0-3 hours post invasion; hpi) were treated with 5% D-Sorbitol
516 to remove remaining schizonts.

517

518 *In vitro* RSAs were conducted as previously described, with minor adaptations (Straimer *et al.*, 2015). Briefly,
519 tightly synchronized 0-3 hpi rings were exposed to a pharmacologically-relevant dose of 700 nM DHA or 0.1%
520 dimethyl sulfoxide (DMSO; vehicle control) for 6 h at 1% parasitemia and 2% hematocrit, washed three times
521 with RPMI medium to remove drug, transferred to fresh 96-well plates, and cultured for an additional 66 h in
522 drug-free medium. Removal of media and resuspension of parasite cultures was performed on a Freedom Evo
523 100 liquid-handling instrument (Tecan). Parasitemias were measured at 72 h by flow cytometry (see below) with
524 at least 50,000 events captured per sample. Parasite survival was expressed as the percentage value of the
525 parasitemia in DHA-treated samples divided by the parasitemia in DMSO-treated samples processed in parallel.
526 We considered any RSA mean survival rates <2% to be ART sensitive.

527

528 Flow cytometry was performed on an BD Accuri™ C6 Plus cytometer with a HyperCyt plate sampling
529 attachment (IntelliCyt), or on an iQue3® Screener Plus cytometer (Sartorius). Cells were stained with 1×SYBR
530 Green (ThermoFisher) and 100 nM MitoTracker DeepRed (ThermoFisher) for 30 min and diluted in 1×PBS prior

531 to sampling. Percent parasitemia was determined as the percentage of MitoTracker⁺ positive and SYBR Green-
532 positive cells. For RSAs, >50,000 events were captured per well.

533

534 **Western blot analysis of K13 expression levels in edited lines**

535 Western blots were performed with lysates from tightly synchronized rings harvested 0-6 h post invasion.
536 Parasite cultures were washed twice in ice-cold 1× phosphate-buffered saline (PBS), and parasites isolated by
537 treatment with 0.05% saponin in PBS. Released parasites were lysed in 4% SDS, 0.5% Triton X-100 and 0.5%
538 PBS supplemented 1× protease inhibitors (Halt Protease Inhibitors Cocktail, ThermoFisher). Samples were
539 centrifuged at 14,000 rpm for 10 min to pellet cellular debris. Supernatants were collected and protein
540 concentrations were determined using the DC protein assay kit (Bio-Rad). Laemmli Sample Buffer (Bio-Rad)
541 was added to lysates and samples were denatured at 90°C for 10 min. Proteins were electrophoresed on
542 precast 4-20% Tris-Glycine gels (Bio-Rad) and transferred onto nitrocellulose membranes. Western blots were
543 probed with a 1:1000 dilution of primary antibodies to K13 (Gnadig *et al.*, 2020) or the loading control ERD2
544 (BEI Resources), followed by a 1:200 dilution of fluorescent StarBright secondary antibodies (Bio-Rad). Western
545 blots were imaged on a ChemiDoc system (Bio-Rad) and band intensities quantified using ImageJ.

546

547 **TaqMan allelic discrimination real-time (quantitative) PCR-based fitness assays**

548 Fitness assays with African *K13*-edited parasite lines were performed by co-culturing isogenic wild-type
549 unedited and mutant edited parasites in 1:1 ratios. Assays were initiated with tightly synchronized trophozoites.
550 Final culture volumes were 3 mL. Cultures were maintained in 12-well plates and monitored every four days
551 over a period of 36 days (18 generations) by harvesting at each time point a fraction of each co-culture for
552 saponin lysis. gDNA was then extracted using the QIAamp DNA Blood Mini Kit (Qiagen). The percentage of the
553 wild-type or mutant allele in each sample was determined in TaqMan allelic discrimination real-time PCR
554 assays. TaqMan primers (forward and reverse) and TaqMan fluorescence-labeled minor groove binder probes
555 (FAM or HEX, Eurofins) are described in **Supplementary file 9**. Probes were designed to specifically detect the
556 K13 R561H, M579I or C580Y propeller mutations. The efficiency and sensitivity of the TaqMan primers was
557 assessed using standard curves comprising 10-fold serially diluted templates ranging from 10 ng to 0.001 ng.
558 Robustness was demonstrated by high efficiency (88-95%) and R^2 values (0.98-1.00). The quantitative accuracy
559 in genotype calling was assessed by performing multiplex qPCR assays using mixtures of wild-type and mutant

560 plasmids in fixed ratios (0:100, 20:80, 40:60, 50:50, 60:40, 80:20, 100:0). Triplicate data points clustered tightly,
561 indicating high reproducibility in the data across the fitted curve ($R^2 = 0.89$ to 0.91).

562

563 Purified gDNA from fitness co-cultures was subsequently amplified and labeled using the primers and probes
564 described in **Supplementary file 9**. qPCR reactions for each sample were run in triplicate. 20 μ L reactions
565 consisted of 1 \times QuantiFAST reaction mix containing ROX reference dye (Qiagen), 0.66 μ M of forward and
566 reverse primers, 0.16 μ M FAM-MGB and HEX-MGB TaqMan probes, and 10 ng genomic DNA. Amplification
567 and detection of fluorescence were carried out on a QuantStudio 3 qPCR machine (Applied Biosystems) using
568 the genotyping assay mode. Cycling conditions were as follows: 30s at 60°C; 5 min at 95°C; and 40 cycles of
569 30s at 95°C and 1 min at 60°C for primer annealing and extension. Every assay was run with positive controls
570 (wild-type or mutant plasmids at different fixed ratios). No-template negative controls (water) in triplicates were
571 processed in parallel. R_n , the fluorescence of the FAM or HEX probe, was normalized to the fluorescence signal
572 of the ROX reporter dye. Background-normalized fluorescence (R_n minus baseline, or ΔR_n) was calculated as a
573 function of cycle number.

574

575 To determine the wild-type or mutant allele frequency in each sample, we first confirmed the presence of the
576 allele by only retaining values where the threshold cycle (C_t) of the sample was less than the no-template control
577 by at least three cycles. Next, we subtracted the ΔR_n of the samples from the background ΔR_n of the no-
578 template negative control. We subsequently normalized the fluorescence to 100% using the positive control
579 plasmids to obtain the percentage of the wild-type and mutant alleles for each sample. The final percentage of
580 the mutant allele was defined as the average of these two values: the normalized percentage of the mutant
581 allele, and 100% minus the normalized percentage of the wild-type allele.

582

583 **eGFP-based fitness assays**

584 Fitness assays with Dd2, RF7^{C580Y} and Cam3.II parasite lines were performed using mixed culture competition
585 assays with an eGFP-positive (eGFP⁺) Dd2 reporter line (Ross *et al.*, 2018). This reporter line uses a *calmodulin*
586 promoter sequence to express high levels of GFP and includes human *dhfr* and *blastcidin S-deaminase*
587 expression cassettes. This line was earlier reported to have a reduced rate of growth relative to parental non-
588 recombinant Dd2, presumably at least in part because of its high levels of GFP expression (Ross *et al.*, 2018;

589 Dhingra *et al.*, 2019). With our Dd2 parasites, *K13*-edited lines were co-cultured in 1:1 ratios with the reporter
590 line. This ratio was adjusted to 10:1 or 100:1 for *fd*-edited RF7^{C580Y} and *mdr2*-edited Cam3.II parasites relative to
591 the eGFP line, given the slower rate of growth with RF7^{C580Y} and Cam3.II. Fitness assays were initiated with
592 tightly synchronized trophozoites in 96-well plates, with 200 µL culture volumes. Percentages of eGFP⁺
593 parasites were monitored by flow cytometry every two days over a period of 20 days (10 generations). Flow
594 cytometry was performed as written above, except that only 100 nM MitoTracker DeepRed staining was used to
595 detect total parasitemias, since SYBR Green and eGFP fluoresce in the same channel.

596

597 **Fitness Costs**

598 The fitness cost associated with a line expressing a given K13 mutation was calculated was calculated relative
599 to its isogenic wild-type counterpart using the following equation:

$$P' = P((1 - x)^n)$$

600 where P' is equal to the parasitemia at the assay endpoint, P is equal to the parasitemia on day 0, n is equal to
601 the number of generations from the assay start to finish, and x is equal to the fitness cost. This equation
602 assumes 100% growth for the wild-type comparator line. For qPCR and GFP-based fitness assays, days 36 and
603 20 were set as the assay endpoints, resulting in the number of parasite generations (n) being set to 18 and 10,
604 respectively.

605

606 **Ethics statement**

607 Health care facilities were in charge of collecting anonymized *P. falciparum* positive cases. Identification of
608 individuals cannot be established. The studies were approved by ethics committees listed in **Supplementary**
609 **file 1**. We note that the sponsors had no role in the study design or in the collection or analysis of the data.
610 There was no confidentiality agreement between the sponsors and the investigators.

611

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630

631 **Competing interests**

632 MW is a former staff member of the World Health Organization. MW alone is responsible for the views
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634 Health Organization. The other authors declare that no competing interests exist.

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917 Legends

918 Figure 1. Frequency and distribution of *K13* alleles in 11 African countries.

919 Map of Africa with pie charts representing the proportions of sequenced samples per country that harbor the
920 *K13* wild-type sequence (3D7 reference), the R561H variant (the most commonly identified mutation, unique to
921 Rwanda; see inset), or another less frequent non-synonymous *K13* mutation. Sample sizes and years of sample
922 collection are indicated. Mutations and numbers of African samples sequenced per country, and prior citations
923 as appropriate, are listed in **Figure 1–source data 1**.

924

925 Figure 1–source data 1. Distribution of *K13* alleles over time in African countries (2011-2019).

926

927 Figure 2. Gene-edited mutant *K13* African parasites display variable levels of RSA survival.

928 (A-D) RSA survival rates for (A) 3D7 (Africa), (B) F32 (Tanzania), (C) UG659 (Uganda), or (D) UG815 (Uganda)
929 *K13* wild-type parental lines and CRISPR/Cas9-edited *K13* R561H, M579I or C580Y mutant clones. Unedited
930 parental lines are described in **Table 1** and **Supplementary file 4**. For 3D7, we also included a *K13* wild-type
931 control (ctrl) line harboring silent shield mutations at the *K13* gRNA cut site. Results show the percentage of
932 early ring-stage parasites (0-3 h post invasion) that survived a 6 h pulse of 700 nM DHA, relative to DMSO-
933 treated parasites assayed in parallel. Percent survival values are shown as means \pm SEM (detailed in **Figure 2–**
934 **source data 1**). Results were obtained from 3 to 8 independent experiments, each performed in duplicate. *P*
935 values were determined by unpaired *t* tests and were calculated for mutant lines relative to the isogenic line
936 expressing wild-type *K13*. ** *P*<0.01; *** *P*<0.001; **** *P*<0.0001.

937

938 Figure 2–source data 1. Ring-stage survival (RSA) assay data for *K13* edited African parasites and 939 controls.

940

941 Figure 2–figure supplement 1. African *K13* mutations result in reduced *K13* protein levels in 3D7 942 parasites.

943 (A) Representative Western blot of parasite extracts probed with an anti-*K13* monoclonal antibody (clone E9)
944 that recognizes full-length *K13* (~85 kDa) and lower molecular weight bands, presumably N-terminal

945 degradation products, as previously reported (Gnadig *et al.*, 2020). Tightly synchronized K13 wild-type, R561H,
946 M579I or C580Y 3D7 parasites were harvested as 0-6 h ring stages. ERD2 was used as a loading control.
947 Experiments were performed on three independent occasions. (B) Quantification of K13 mutant protein levels
948 versus K13 wild-type protein levels across independent replicates, performed using ImageJ, with all protein
949 levels normalized to the ERD2 loading control. Western blots revealed reduced levels of K13 protein in the three
950 mutant lines relative to wild-type 3D7 parasites. Results are shown as means \pm SEM. WT, wild-type.

951

952 **Figure 2—figure supplement 1—source data 1. Raw figure files for K13 Western blots performed on 3D7**
953 **parasites.**

954

955 **Figure 3. K13 mutations cause differential impacts on *in vitro* growth rates across gene-edited African**
956 **strains.**

957 (A-D) Percentage of mutant allele relative to the wild-type allele over time in (A) 3D7, (B) F32, (C) UG659, and
958 (D) UG815 parasite cultures in which K13 mutant clones were co-cultured at 1:1 starting ratios with isogenic
959 K13 wild-type controls over a period of 36 days. Results, shown as means \pm SEM, were obtained from 2 to 5
960 independent experiments, each performed in duplicate. Values are provided in **Figure 3—source data 1**. (E)
961 The percent reduction in growth rate per 48 h generation, termed the fitness cost, is presented as mean \pm SEM
962 for each mutant line relative to its isogenic wild-type comparator. (F) Fitness costs for mutant lines and isogenic
963 wild-type comparators plotted relative to RSA survival values for the same lines.

964

965 **Figure 3—source data 1. Fitness assay data for K13 edited African parasite lines and controls.**

966

967 **Figure 4. The K13 C580Y allele has progressively outcompeted all other alleles in Cambodia.**

968 (A-D) Stacked bar charts representing the percentage of sequenced samples expressing the K13 wild-type
969 allele or individual variants, calculated based on the total number of samples (listed in parentheses) for a given
970 period. Sample collection was segregated into four regions in Cambodia (detailed in **Figure 4—figure**
971 **supplement 1**). All K13 mutant samples harbored a single non-synonymous nucleotide polymorphism.
972 Mutations and numbers of Cambodian samples sequenced per region/year, including prior citations as
973 appropriate, are listed in **Figure 4—source data 1**.

974

975 **Figure 4—source data 1. Distribution of *K13* alleles over time in Cambodia (2001-2017).**

976

977 **Figure 4—figure supplement 1. Regions of sample collection in Cambodia for *K13* sequencing.**

978 Map depicting the four regions of Cambodia (western, northern, eastern, and southern) in which samples were
979 collected between 2001 and 2017 for *K13* genotyping. Genotyping data are presented in **Figure 4**.

980

981 **Figure 5. Southeast Asian *K13* mutations yield elevated RSA survival and minor impacts on *in vitro***
982 **growth in gene-edited parasite lines.**

983 (A, B) RSA survival rates for Dd2 (Indochina) and Cam3.II (Cambodia) *P. falciparum* parasites expressing wild-
984 type or mutant *K13*. Gene-edited parasites were generated using CRISPR/Cas9 or zinc-finger nucleases.
985 Control (ctrl) lines express silent shield mutations at the *K13* gRNA cut site. Parental lines are described in
986 **Table 1** and **Supplementary file 4**. Results show the percentage of early ring-stage parasites (0-3 h post
987 invasion) that survived a 6 h pulse of 700 nM DHA, relative to DMSO-treated parasites processed in parallel.
988 Percent survival values are shown as means \pm SEM (detailed in **Figure 5—source data 1**). Results were
989 obtained from 3 to 13 independent experiments, each performed in duplicate. *P* values were determined by
990 unpaired *t* tests and were calculated for mutant lines relative to the isogenic line expressing wild-type *K13*.
991 *** $P < 0.001$; **** $P < 0.0001$. (C) Percent reductions in growth rate per 48 h generation, expressed as the fitness
992 cost, for each Dd2 mutant line relative to the Dd2^{WT} line. Fitness costs were determined by co-culturing a
993 Dd2^{eGFP} reporter line with either the Dd2 *K13* wild-type parental line (Dd2^{WT}) or gene-edited *K13* mutant lines.
994 Co-cultures were maintained for 20 days and percentages of eGFP⁺ parasites were determined by flow
995 cytometry (see **Figure 5—source data 2** and **Figure 5—figure supplement 1**). Fitness costs were initially
996 calculated relative to the Dd2^{eGFP} reporter line (**Figure 5—figure supplement 1**) and then normalized to the
997 Dd2^{WT} line. Mean \pm SEM values were obtained from three independent experiments, each performed in
998 triplicate. (D) Fitness costs for *K13* mutant lines, relative to the Dd2^{WT} line, were plotted against their
999 corresponding RSA survival values.

1000

1001 **Figure 5—source data 1. Ring-stage survival (RSA) assay data for *K13* edited SE Asian parasites and**
1002 **controls (Dd2 and Cam3.II strains).**

1003

1004 **Figure 5—source data 2. Fitness assay data (percent eGFP+ parasites) for *K13* edited Dd2 parasites and**
1005 **parental control.**

1006

1007 **Figure 5—figure supplement 1. Southeast Asian *K13* mutations result in minor in vitro growth defects in**
1008 **Dd2 parasites, with the exception of the C580Y and P553L mutations.**

1009 (A) Percentage of eGFP⁺ parasites over time in parasite cultures in which an eGFP-expressing Dd2 line was co-
1010 cultured in a 1:1 mixture with either the Dd2 *K13* WT parental line (Dd2^{WT}) or individual Dd2 gene-edited *K13*
1011 mutant lines. Co-cultures were maintained over a period of 20 days, and the percentage of eGFP⁺ parasites in
1012 each mix was determined by flow cytometry. Data are shown as means ± SEM. Results were obtained from
1013 three independent experiments, each performed in triplicate. (B) Percent reduction in growth rate per 48 h
1014 generation, termed the fitness cost, for Dd2^{WT} and *K13*-edited mutant lines relative to the Dd2^{eGFP} line. Results
1015 are shown as means ± SEM.

1016

1017 **Figure 6. Thai isolates expressing mutant *K13* display variable RSA survival rates.**

1018 RSA survival rates for (A-E) *K13*-edited Thai isolates and (F) *K13* E252Q unedited Thai lines, shown as means
1019 ± SEM (detailed in **Figure 6—source data 1**). Results were obtained from 3 to 7 independent experiments, each
1020 performed in duplicate. *P* values were determined by unpaired *t* tests and were calculated for mutant lines
1021 relative to the isogenic line expressing wild-type *K13*. * *P*<0.05; ** *P*<0.01; *** *P*<0.001.

1022

1023 **Figure 6—source data 1. Ring-stage survival (RSA) assay data for *K13* edited Thai parasites and controls.**

1024

1025 **Figure 7. Ferredoxin (fd) and multidrug resistance protein 2 (mdr2) mutations do not impact RSA**
1026 **survival or in vitro growth rates in *K13* C580Y parasites.**

1027 RSA survival rates for (A) RF7^{C580Y} parasite lines expressing the fd variant D193Y (parent), this variant plus
1028 silent shield mutations (edited control), or fd D193 (edited revertant), and (C) Cam3.II^{C580Y} parasite lines
1029 expressing the mdr2 variant T484I (parent), this variant plus silent shield mutations (edited control), or mdr2

1030 T484 (edited revertant). Parental lines are described in **Table 1** and **Supplementary file 4**. Mean \pm SEM
1031 survival rates were generated from three independent experiments, each performed in duplicate. (B, D) *In vitro*
1032 eGFP-based fitness assays performed with (B) *fd* and (D) *mdr2* RF7^{C580Y} or Cam3.II^{C580Y} edited lines,
1033 respectively. Competitive growth assays were seeded with individual lines plus the Dd2^{eGFP} reporter line at a
1034 10:1 starting ratio. Results show the percentage of eGFP⁺ parasites over time. Co-cultures were maintained
1035 over a period of 24 days (*fd*-edited lines) or 30 days (*mdr2*-edited lines) and percentages of eGFP⁺ parasites
1036 were determined by flow cytometry. Results were obtained from 2-3 independent experiments, each performed
1037 in triplicate, and are shown as means \pm SEM. All values are provided in **Figure 7–source data 1–3**.

1038

1039 **Figure 7–source data 1. Ring-stage survival (RSA) assay data for *fd* and *mdr2* edited parasites and**
1040 **controls.**

1041

1042 **Figure 7–source data 2. Fitness assay data (percent eGFP+ parasites) for RF7 *fd* edited parasites and**
1043 **parental control.**

1044

1045 **Figure 7–source data 3. Fitness assay data (percent eGFP+ parasites) for Cam3.II *mdr2* edited parasites**
1046 **and parental control.**

1047

1048 **Figure 7–figure supplement 1. Ferredoxin (*fd*) and multidrug resistance protein 2 (*mdr2*) mutations do**
1049 **not impact RSA survival or *in vitro* growth in K13 C580Y parasites.**

1050 (A, B) Ring-stage survival assays (RSAs) performed on *fd* and *mdr2* edited lines and parental controls (RF7^{C580Y}
1051 and Cam3.II^{C580Y}, respectively). Results show RSA survival rates across a range of DHA concentrations.
1052 Survival rates were calculated relative to DMSO-treated parasites processed in parallel. Results were obtained
1053 from three independent experiments, each performed in duplicate. Data are shown as means \pm SEM. (C, D) *In*
1054 *vitro* eGFP-based fitness assays performed with (C) *fd* and (D) *mdr2* RF7^{C580Y} or Cam3.II^{C580Y} edited lines,
1055 respectively. Competitive growth assays were seeded with individual lines plus the Dd2^{eGFP} reporter line at a
1056 100:1 starting ratio. Results show the percentage of eGFP⁺ parasites over time. Co-cultures were maintained
1057 over a period of 24 days (*fd*-edited lines) or 30 days (*mdr2*-edited lines) and percentages of eGFP⁺ parasites

1058 were determined by flow cytometry. Results were obtained from 2 to 3 independent experiments, each
1059 performed in triplicate, and are shown as means \pm SEM.

1060

1061 **Supplementary Files**

1062 **Supplementary file 1. Sample information and approval from within-country ethics committees for K13**
1063 **genotyping data.**

1064

1065 **Supplementary file 2. CRISPR/Cas9 strategy for editing the K13 locus.**

1066 All-in-one plasmid approach used for CRISPR/Cas9-mediated *K13* gene editing, consisting of a *K13*-specific
1067 donor template for homology-directed repair, a *K13*-specific gRNA expressed from the *U6* promoter, a Cas9
1068 cassette with expression driven by the *calmodulin* (*cam*) promoter, and a selectable marker (human *dhfr*,
1069 conferring resistance to the antimalarial WR99210 that inhibits *P. falciparum dhfr*). The Cas9 sequence was
1070 codon-optimized for improved expression in *P. falciparum*. Donors coding for specific mutations of interest (e.g.,
1071 K13 C580Y, red star) were generated by site-directed mutagenesis of the K13 wild-type donor sequence. Green
1072 bars indicate the presence of silent shield mutations that were introduced to protect the edited locus from further
1073 cleavage. The lightning bolt indicates the location of the cut site in the genomic target locus. Primers used for
1074 cloning and final plasmids are described in **Supplementary files 7 and 8**, respectively.

1075

1076 **Supplementary file 3. Crystal structure of K13 propeller domain showing positions of mutated residues.**

1077 (A, B) Top and (C, D) side views of the crystal structure of the K13 propeller domain (PDB ID: 4YY8),
1078 highlighting residues of interest (F446I, orange; R539T, dark blue; I543T, purple; P553L, pink; R561H, dark
1079 turquoise; P574L, light turquoise; M579I medium blue; C580Y, red). Structures shown in (A) and (C) show wild-
1080 type residues while (B) and (D) show mutated residues.

1081

1082 **Supplementary file 4. Geographic origin and drug resistance genotypes of Plasmodium falciparum**
1083 **clinical isolates and reference lines employed in this study.**

1084

1085 **Supplementary file 5. Transgenic *Plasmodium falciparum* lines generated in this study.**

1086

1087 **Supplementary file 6. CRISPR/Cas9 strategy for editing the *ferredoxin (fd)* and *multidrug resistance***
1088 ***protein 2 (mdr2)* loci.**

1089 All-in-one plasmid approaches used for CRISPR/Cas9-mediated editing of (A) the *ferredoxin (fd)* locus or (B) the
1090 *multidrug resistance protein 2 (mdr2)* locus. Plasmids consisted of a *fd* (A) or *mdr2* (B) specific donor template
1091 for homology-directed repair, a gene-specific gRNA expressed from the *U6* promoter, a Cas9 cassette with
1092 expression driven by the *cam* promoter, and a selectable marker (human *dhfr*, conferring resistance to
1093 WR99210). Donors coding for specific mutations of interest (*fd* D193Y or *mdr2* T484I) were generated by site-
1094 directed mutagenesis of the wild-type donor sequences. Red bars indicate the presence of silent shield
1095 mutations used to protect edited loci from further cleavage. Primers used for cloning and final plasmids are
1096 described in **Supplementary files 7 and 8**, respectively.

1097

1098 **Supplementary file 7. Oligonucleotides used in this study.**

1099

1100 **Supplementary file 8. Description of gene-editing plasmids generated in this study.**

1101

1102 **Supplementary file 9. Real-Time PCR (qPCR) primers and probes used in this study.**

1103 **Table 1. *Plasmodium falciparum* lines employed herein.**

Parasite	Origin	Year	K13	Resistance
3D7 ^{WT}	Africa	1981	WT	--
F32 ^{WT}	Tanzania	1982	WT	--
UG659 ^{WT}	Uganda	2007	WT	CQ, SP
UG815 ^{WT}	Uganda	2008	WT	CQ, SP
Dd2 ^{WT}	Indochina	1980	WT	CQ, MFQ, SP
Cam3.II ^{WT}	Cambodia	2010	WT	CQ, SP
CamWT ^{C580Y}	Cambodia	2010	C580Y	ART, CQ, SP
RF7 ^{C580Y}	Cambodia	2012	C580Y	ART, CQ, PPQ, SP
Thai1 ^{WT}	Thailand	2003	WT	CQ, SP
Thai2 ^{WT}	Thailand	2004	WT	CQ, MFQ, SP
Thai3 ^{WT}	Thailand	2003	WT	CQ, SP
Thai4 ^{WT}	Thailand	2003	WT	CQ, SP
Thai5 ^{WT}	Thailand	2011	WT	CQ, SP
Thai6 ^{E252Q}	Thailand	2008	E252Q	ART (low), CQ, MFQ, SP
Thai7 ^{E252Q}	Thailand	2010	E252Q	ART (low), CQ, MFQ, SP

ART, artemisinin; CQ, chloroquine; MFQ, mefloquine; PPQ, piperazine; SP, sulfadoxine/pyrimethamine; WT, wild type.

1104

Figure 1

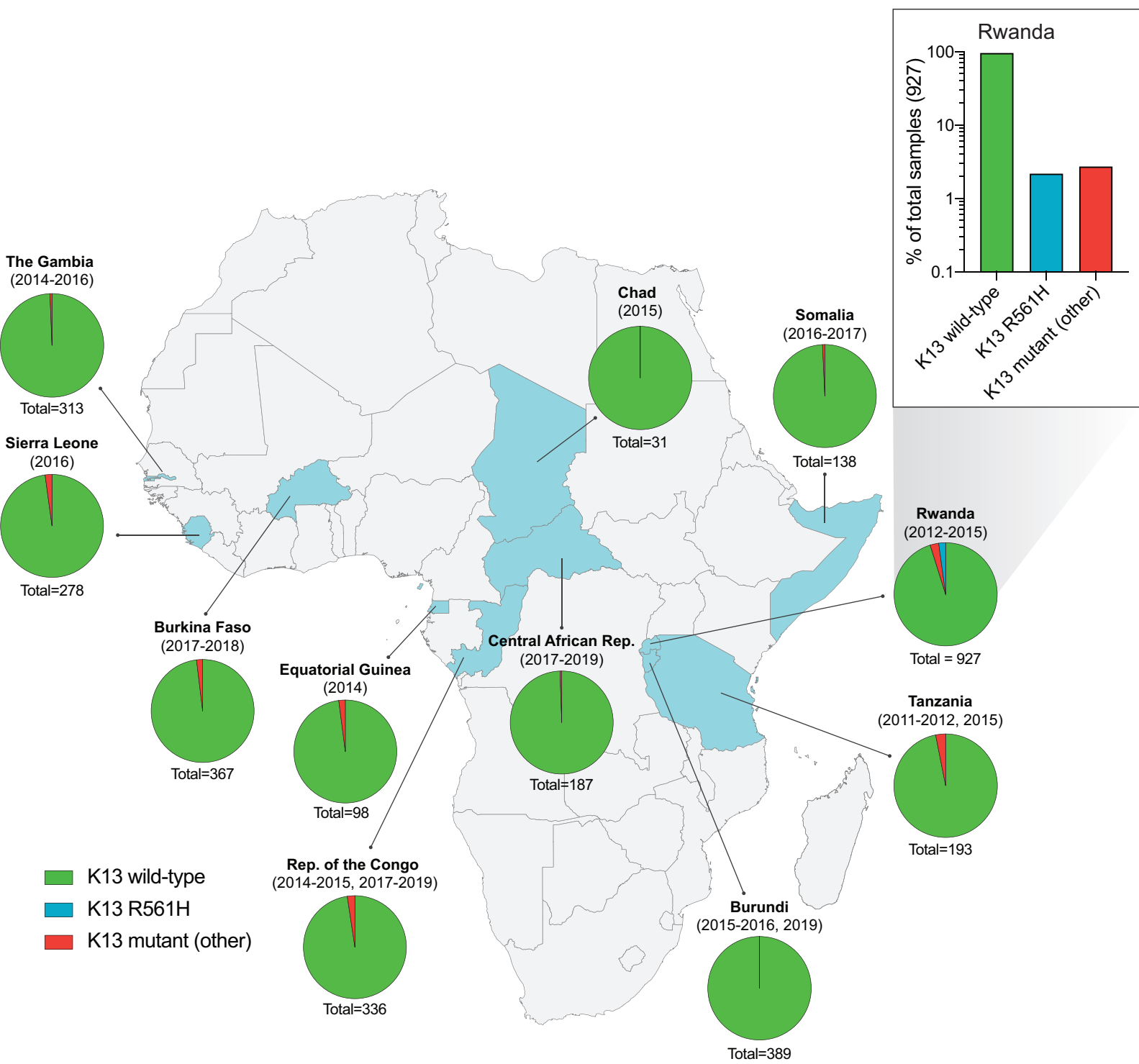


Figure 2

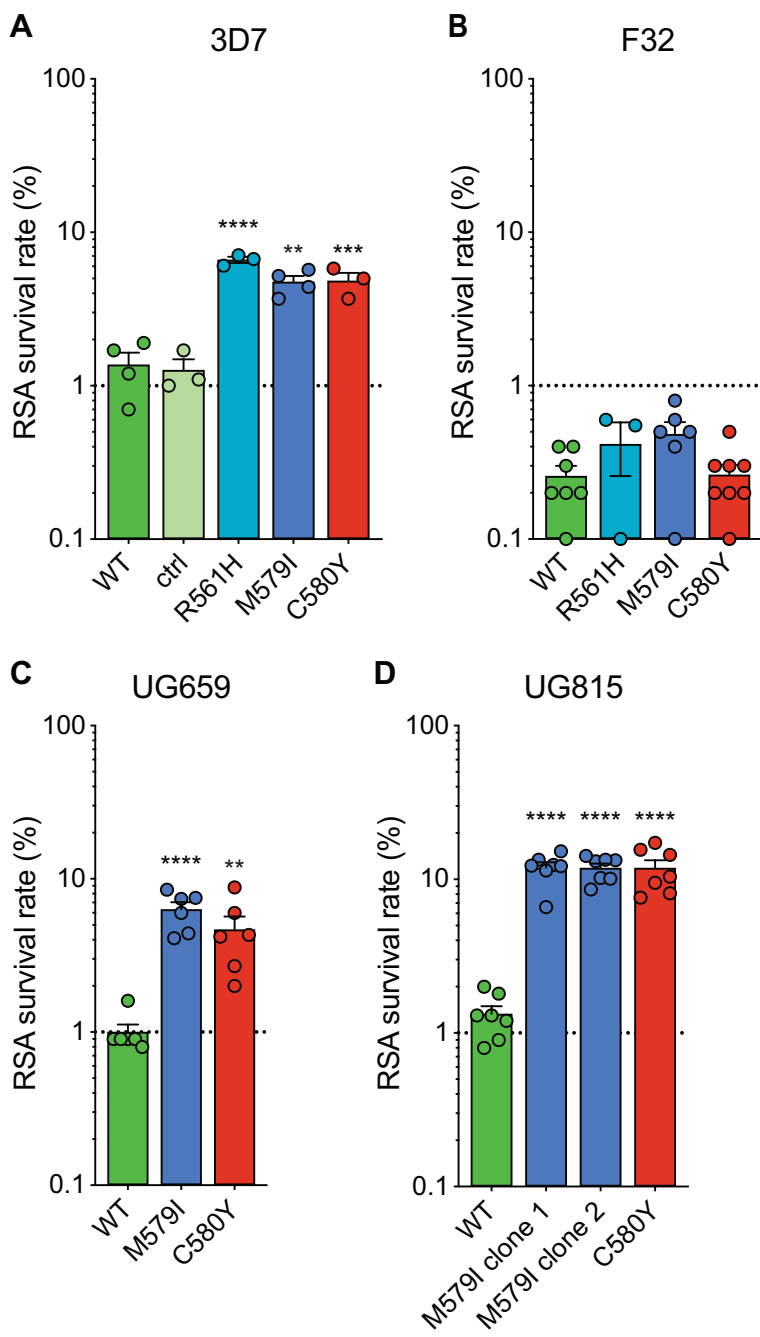


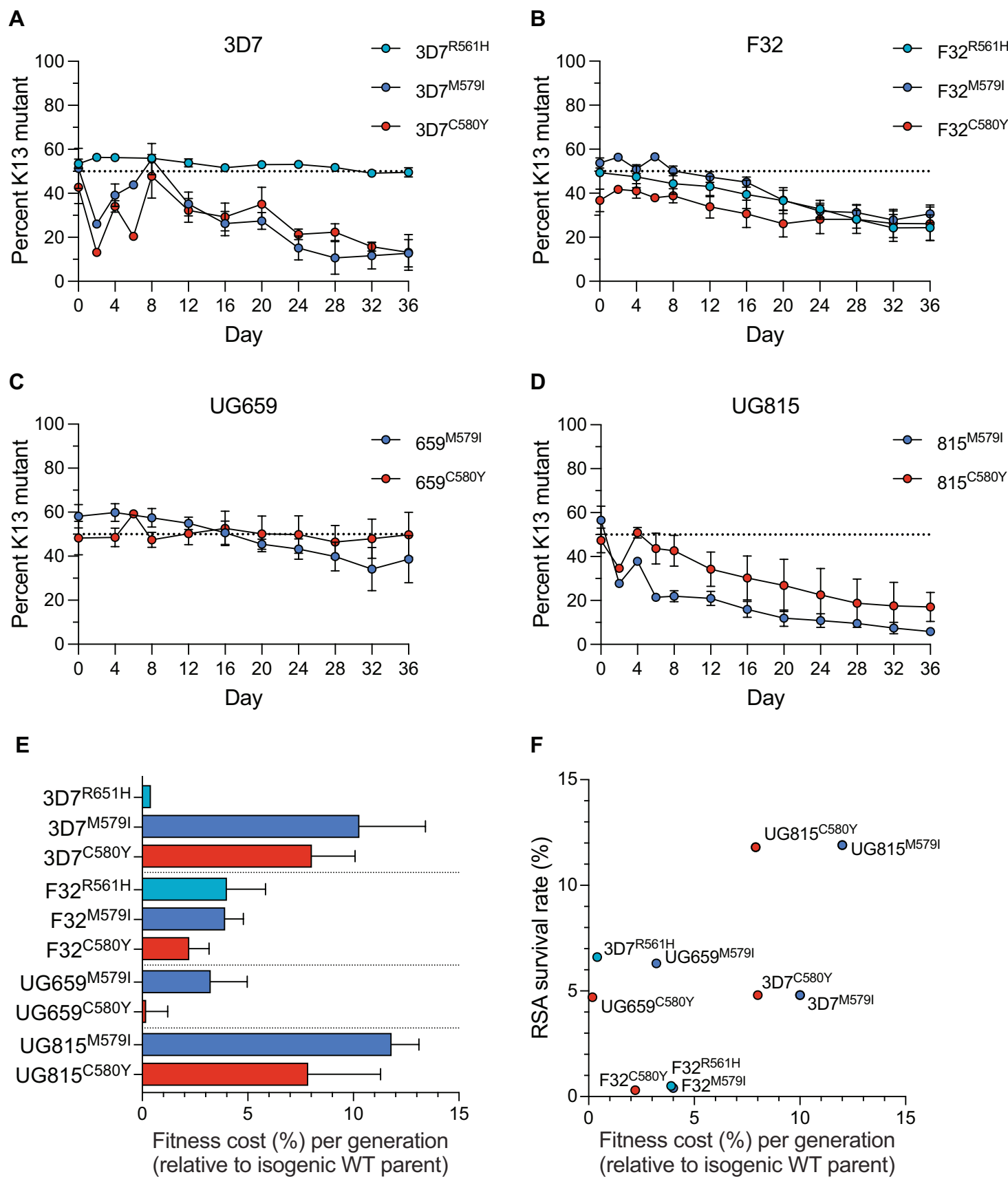
Figure 3

Figure 4

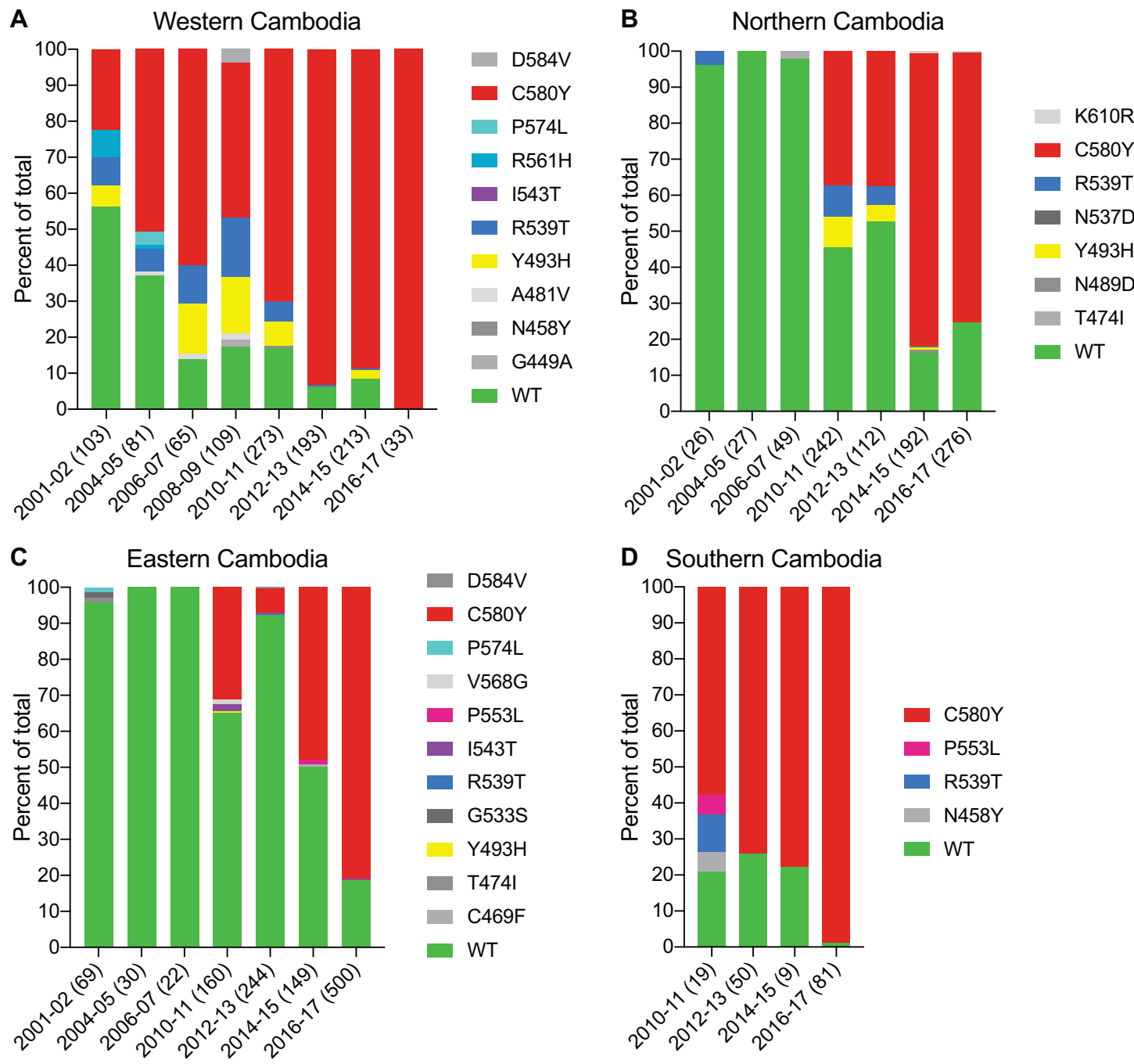


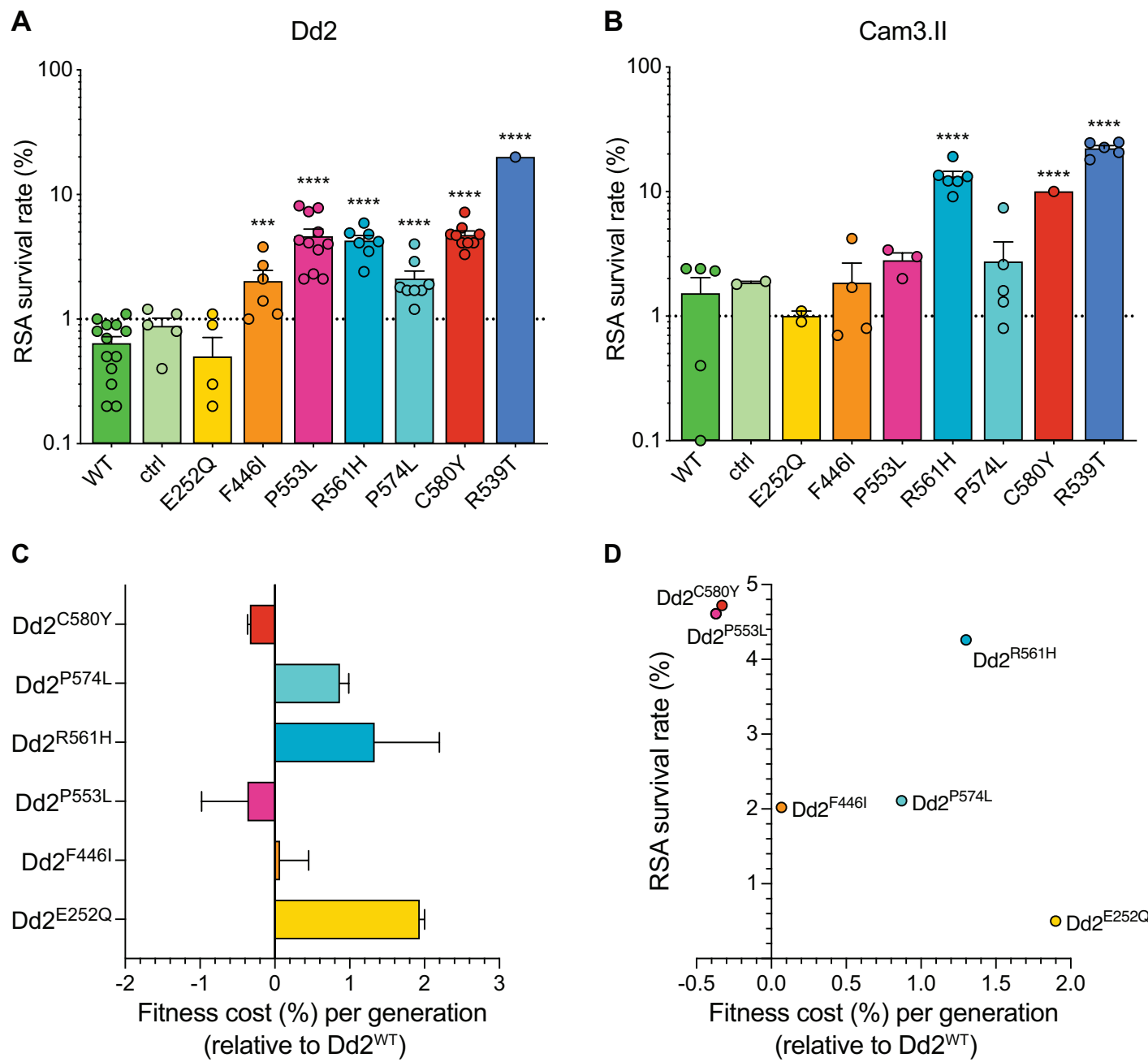
Figure 5

Figure 6

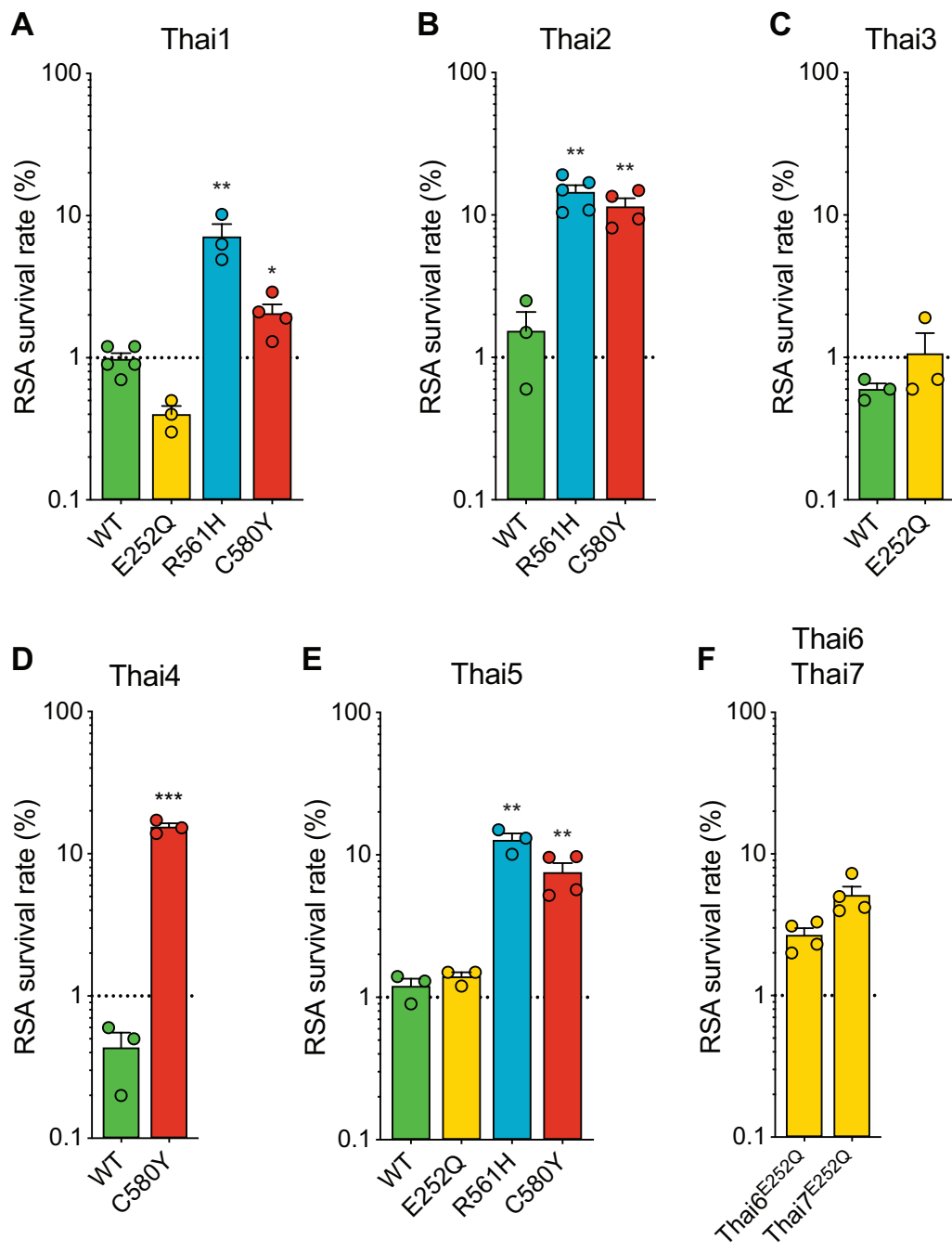


Figure 7

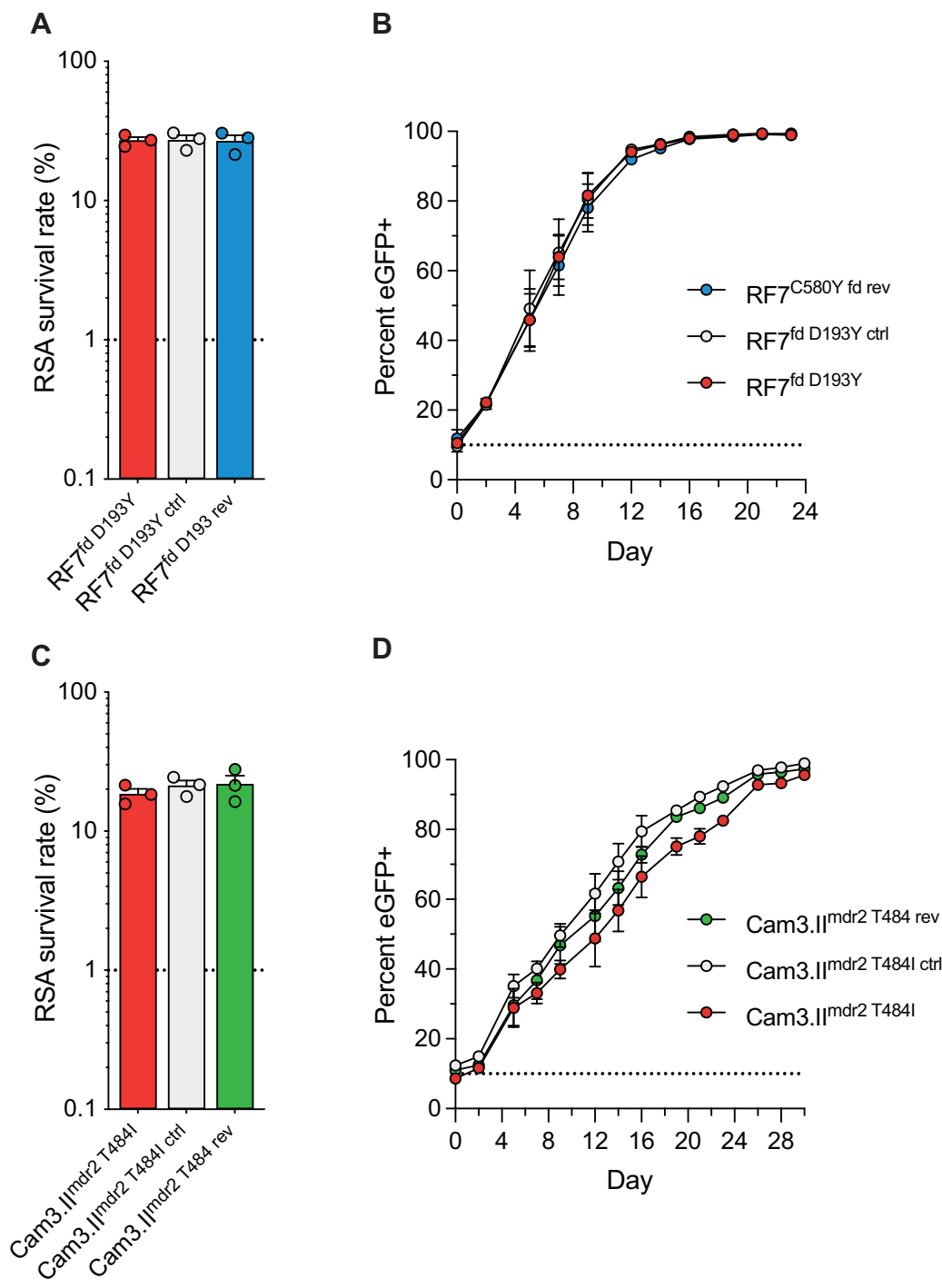


Figure 2—figure supplement 1

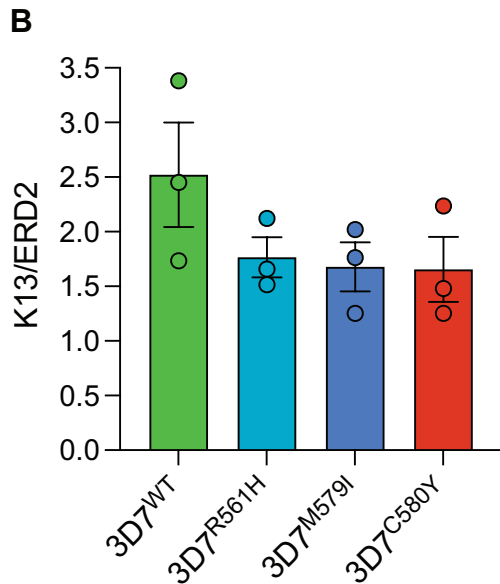
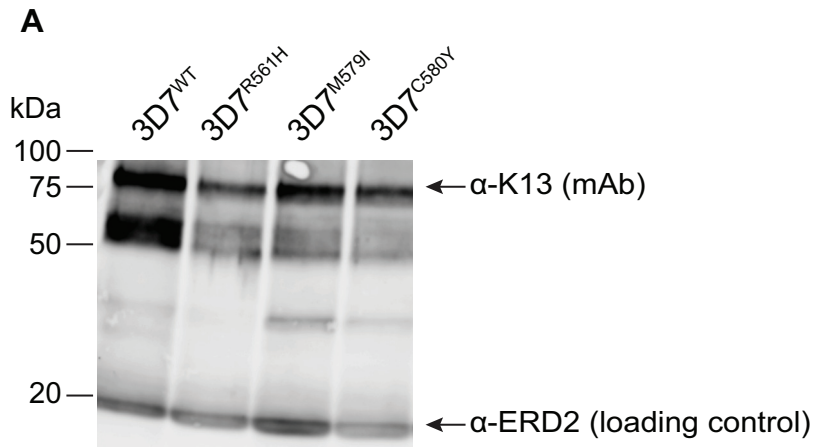
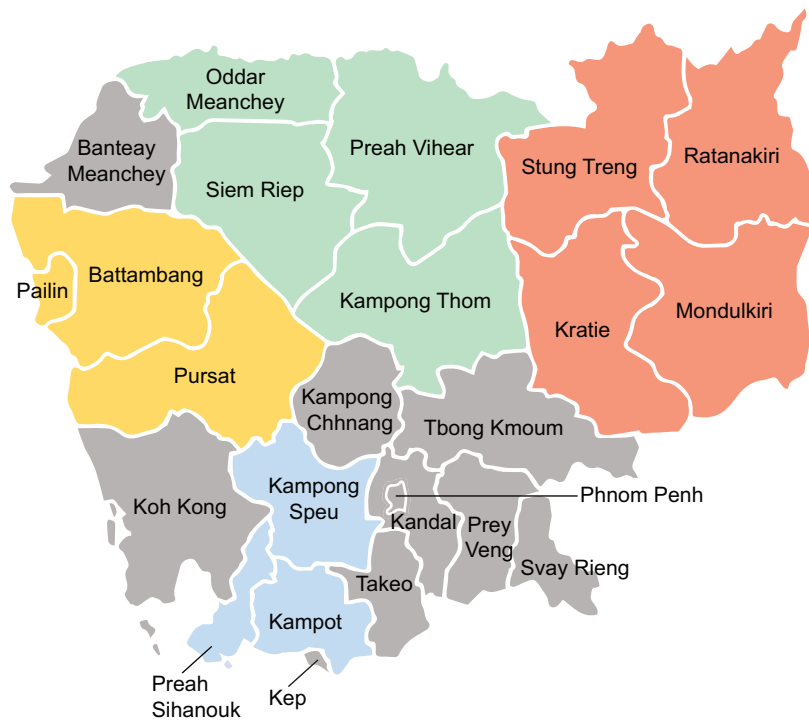


Figure 4—figure supplement 1



Western Cambodia

Battambang
Pailin
Pursat

Northern Cambodia

Oddar Meanchey
Preah Vihear
Kampong Thom
Siem Riep

Eastern Cambodia

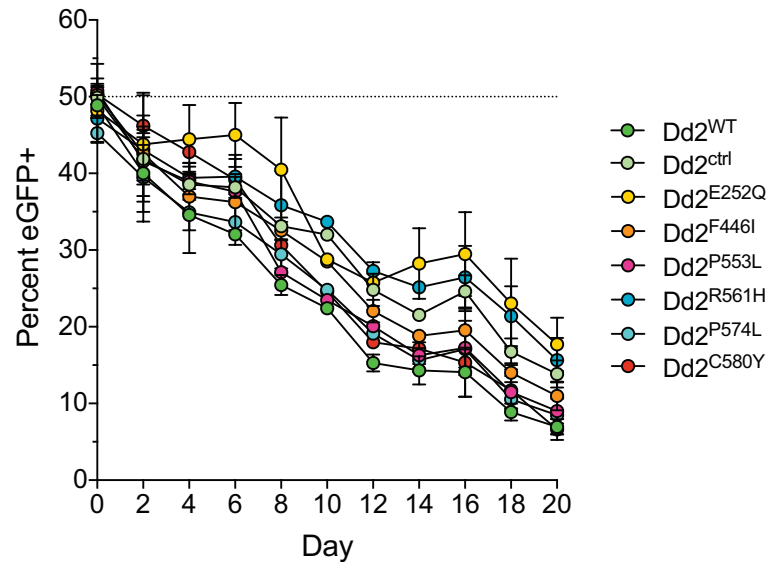
Stung Treng
Ratanakiri
Mondulakiri
Kratie

Southern Cambodia

Kampong Speu
Kampot
Preah Sihanouk

Figure 5—figure supplement 1

A



B

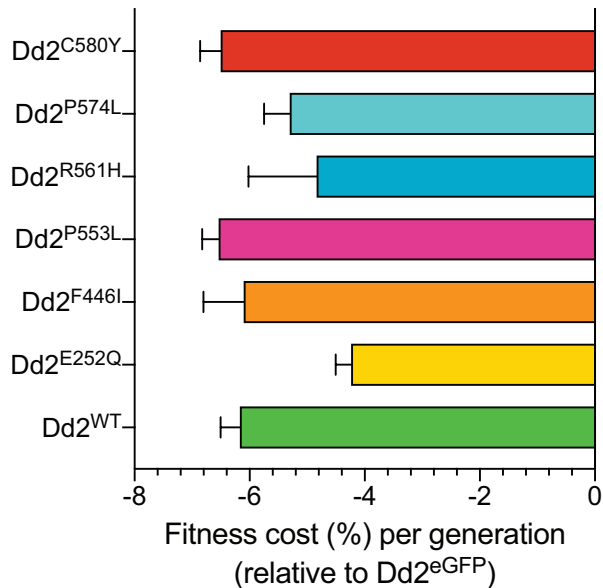


Figure 7–figure supplement 1

