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Summary (50 words)

Amino acid deprivation from reduced hemoglobin degradation in *Pfkelch13* artemisinin-resistant parasites reduces fitness. In this issue of Cell Host & Microbe, Mesén-Ramírez et *al.* (2021) decipher the role of nutrient permeable channel activity within the parasitophorous vacuolar membrane to compensate for this fitness cost in asexual blood-stage *Plasmodium falciparum* parasites.

Main text (995 words)

Malaria, caused by the protozoan parasite *Plasmodium falciparum,* is a major public health issue and a potentially fatal disease in tropical regions. Currently, strategies for malaria control and treatment rely on mosquito vector control and the prompt and effective management of malaria cases. However, *P. falciparum* resistance to antimalarial drugs remains a permanent threat that jeopardizes efforts and gains made in reducing the global burden of malaria. This ‘sword of Damocles’ seems to be an inevitable outcome of the drugs' widespread use(White, 2004). Over the last century, nearly all the antimalarial drugs deployed worldwide have led to the selection and spread of drug-resistant parasites. This is the case for artemisinin derivatives, the most potent antimalarial drug and the cornerstone of current first-line artemisinin-based combination therapies (ACTs)(Menard and Dondorp, 2017). Although artemisinins are active against a large range of intraerythrocytic developmental stages, their usefulness is curtailed by ring-stage resistance(Mok et al., 2015).

Seminal genetic studies have demonstrated that *P. falciparum* resistance to antimalarial drugs (*i.e.* chloroquine, antifolates, mefloquine or piperaquine) is associated with the spontaneous arising of mutations in genes or changes in the copy number of genes encoding proteins affecting drug influx/efflux or drug binding affinity(Ross and Fidock, 2019). With regard to artemisinins, mutations in a *kelch* gene located on chromosome 13 (*Pfkelch13*) have been shown to be a major determinant of both *in vitro* and *in vivo* artemisinin resistance(Ariey et al., 2014; Straimer et al., 2015). These mutations mediate artemisinin

resistance via a reduced hemoglobin endocytosis and catabolism in rings (the youngest blood-stage parasite), resulting in lowered levels of free ferrous-protoporphyrin IX [Fe(II)PPIX] available to activate artemisinin and an enhanced parasite capacity to remove damaged proteins. As a consequence, artemisinin-resistant *Pfkelch13* mutant parasites are reported to be less fit compared with wild-type parasites(Ross and Fidock, 2019).

In this issue of Cell Host & Microbe, Mesén-Ramírez and colleagues unveil a role for the activity of nutrient permeable channels (NPCs) located in the parasite vacuole membrane (PVM, an enveloping membrane that surrounds the parasite). The NPC activity modulates the acquisition of nutrients (including glucose and amino acids from the host's serum) and in some cases apparently the passage of antimalarial drugs. NPC activity also appears to compensate for fitness costs associated with artemisinin resistance.

Based on engineered parasites expressing different levels of EXP1(Mesen-Ramirez et al., 2019), this study provides compelling evidence that EXP1 is a major contributor to NPC activity. EXP1 expression, already active in the ring stages when artemisinin resistance occurs, directly influences the access of nutrients through the NPCs. Using *in vitro* susceptibility assays with parasites expressing reduced versus normal levels of EXP1, these authors also assessed whether NCPs could regulate the access of antimalarial drugs into the parasite. Results provided evidence that the NPCs might act as a conduit for several antimalarial drugs, depending on their physicochemical properties. Small water-soluble molecules appeared to reach the parasite via the NPCs whereas hydrophobic or bulky molecules appeared to cross the PVM independent of the NPCs. NPC activity was observed to facilitate the access of the 4-aminoquinolines (4-AQ) chloroquine, amodiaquine, and mefloquine, as well as the endoperoxide dihydroartemisinin. The amount of drug reaching the parasite also correlated with the level of activity of EXP1. However, for some other antimalarial drugs like lumefantrine, an aryl amino alcohol, the passage through the PVM was not influenced by the level of EXP1 expression.

Of interest, Mesén-Ramírez et *al.* noted that, in contrast to the other tested 4-AQs, parasites expressing low levels of EXP1 were highly susceptible to piperaquine (a companion drug combined with dihydroartemisinin in an ACT), suggesting that piperaquine reaches the parasite independent of the NPCs. Further analysis confirmed that elevated NPC activity increased amino acid availability from the host's serum rather than the passage of piperaquine, highlighting the dual mode of action of this molecule: inhibition of hemozoin formation as observed with other 4-AQs, and the inhibition of hemoglobin proteolysis leading to a reduced acquisition of hemoglobin-derived amino acids. This finding might suggest that

defining whether antimalarial drugs can passage through the NPC should be taken into account in the selection of lead compounds and designing new combinations such as triple ACTs(van der Pluijm et al., 2021).

Finally, Mesén-Ramírez et *al.* provide convincing data on how *P. falciparum* ring stages resistant to artemisinin manage the shortage of hemoglobin-derived amino acids that is a consequence of mutant *Pfkelch13* causing reduced hemoglobin endocytosis. Their data suggest that compensatory mechanisms in artemisinin-resistant parasites might involve the increased expression of EXP1 and thus the supply of amino acids through the NPC. Increased EXP1 expression was found to restore the growth of artemisinin-resistant parasites to levels observed with isogenic *Pfkelch13* wild-type parasites (Figure 1, panel A). This mechanism is reminiscent of how parasites respond to low amino acid conditions. Transcriptomic and proteomic analyses of field samples obtained from Southeast Asian malaria patients with known artemisinin resistance provided further evidence that the change in expression of EXP1 might be a natural mechanism to compensate for the fitness cost caused by hemoglobin-derived amino acid restriction. This might have facilitated the rapid spread of *Pfkelch13* C580Y mutant parasites in the Greater Mekong Subregion over the last decade(Imwong et al., 2020) (Figure 1, panel B).

This study sheds light on how *P. falciparum* malaria parasites can acquire effective compensatory mechanisms that counterbalance fitness costs linked to genetic changes associated with drug resistance. This work also raises several core questions. Which regulatory factors control the expression of EXP1 (*i.e*. a transcriptional regulator, DNA methylation, or post-translational modification) and how? Is EXP1 expression adaptive or heritable? Do other biological pathways such as autophagy, eukaryotic initiation factor 2α -IF2α- or PfMAF1-associated nutrient and stress response pathways participate in reducing the fitness cost of artemisinin resistance? Understanding mechanisms that mitigate the fitness cost associated with artemisinin resistance might help define further strategies aiming at extending the usefulness of artemisinin or developing a better rationale for combining partner drugs with artemisinin in triple ACTs with the ultimate intent of eliminating malaria.

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Figure legend

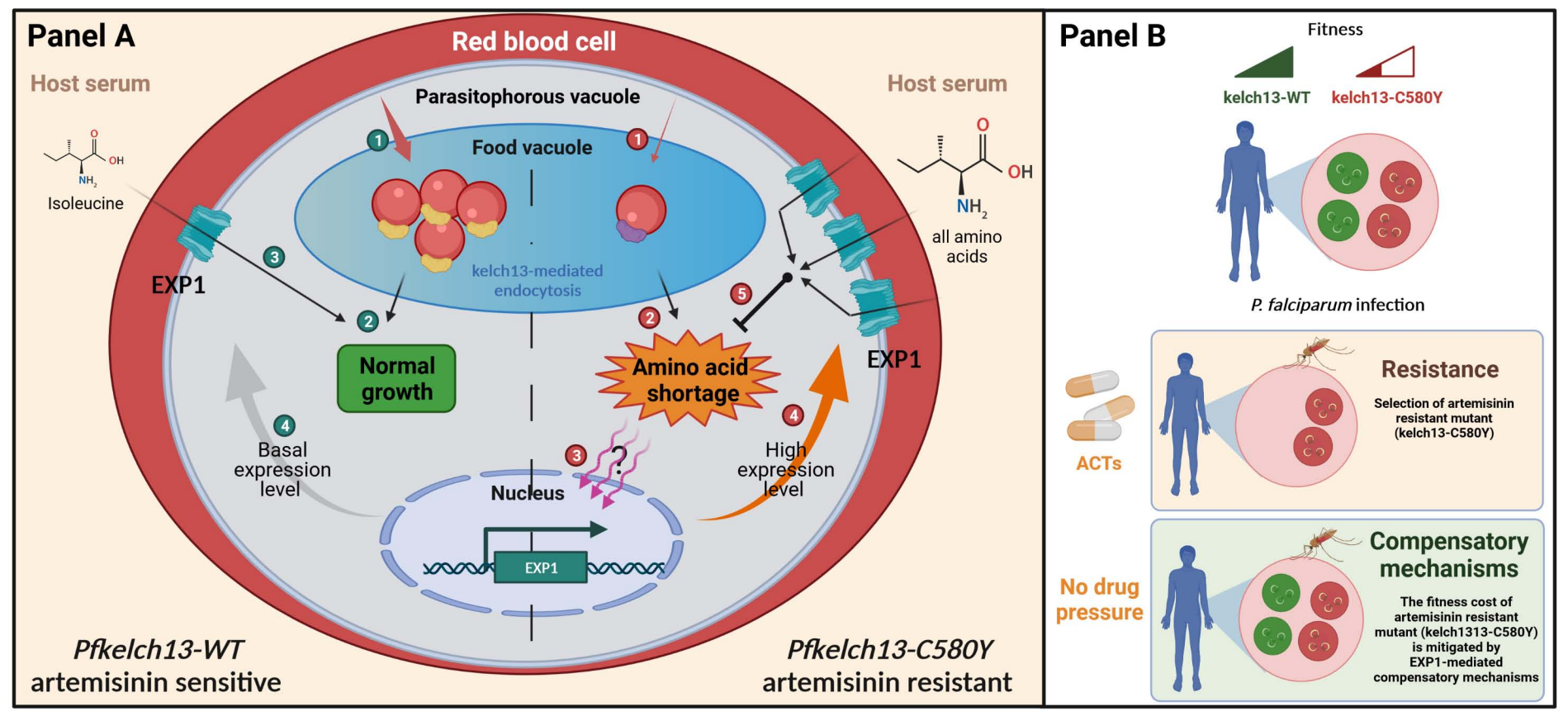
Title. Compensatory mechanism in *P. falciparum* artemisinin resistance: mechanisms and consequences.

Panel A. Biological mechanisms (individual level).

Pfkelch13-WT (left side): 1) Hemoglobin uptake is mediated by Pfkelch13-WT protein by endocytosis; 2) Hemoglobin digestion provided amino acids to sustain continuous and optimal growth (optimal transition rate from ring- to trophozoite-stages) and 3-4) Isoleucine and nutrients are obtained from the host's serum through the nutrient permeable channel activity (basal production of EXP1 protein).
Pfkelch13-C580Y (right side): 1) Hemoglobin uptake is reduced in Pfkelch13 C580Y mutant parasites; 2) Amino acid deprivation from the reduced hemoglobin degradation drives a fitness cost; 3) In response to amino acid shortage, EXP1 is highly expressed under the control of unknown regulatory factors; 4) Increased production of EXP1 proteins increases the supply of amino acids from the host's serum through the nutrient permeable channel located in the parasitophorous vacuolar membrane and 5) Increased nutrient permeable channel activity mitigates the fitness cost associated with artemisinin resistance in asexual blood stage *Plasmodium falciparum* parasites.

Panel B. Epidemiological consequences (population level).

P. falciparum infections treated with ACT lead to the selection of artemisinin resistant parasites. Only *Pfkelch13*-C580Y mutant parasites are capable to survive to the drug exposure despite its reduced fitness compared to *Pfkelch13*-WT parasites.
When parasites are not exposed to artemisinin (like asymptomatic infections), change in expression of EXP1 in *Pfkelch13*-C580Y mutant parasites compensate for the fitness cost caused by hemoglobin-derived amino acid restriction. This natural compensatory mechanism allows *Pfkelch13*-C580Y mutant parasites population to compete with *Pfkelch13*-WT parasites population and secure the transmission of its gametocytes (sexual blood stages) to new hosts.


Panel A
Red blood cell
Host serum
Host serum
Parasitophorous vacuole
Food vacuole
Isoleucine
all amino acids
EXP1
EXP1
kelch13-mediated endocytosis
Normal growth
Amino acid shortage
Basal expression level
High expression level
Nucleus
EXP1
Pfkelch13-WT
artemisinin sensitive
Pfkelch13-C580Y
artemisinin resistant
Panel B
Fitness
kelch13-WT
kelch13-C580Y
P. falciparum infection
ACTs
Resistance
Selection of artemisinin resistant mutant (kelch13-C580Y)
No drug pressure
Compensatory mechanisms
The fitness cost of artemisinin resistant mutant (kelch1313-C580Y) is mitigated by EXP1-mediated compensatory mechanisms