Title

Probing Translational Regulation by the Malaria Parasite Plasmodium falciparum: Applying a Novel In Vitro Assay to Identify Genetic Determinants of Regulation and Identify Small Molecules Exploiting P. falciparum Translation as a Drug Target

Author

Sheridan, Christine Moore

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Probing Translational Regulation by the Malaria Parasite Plasmodium falciparum: Applying a Novel In Vitro Assay to Identify Genetic Determinants of Regulation and Identify Small Molecules Exploiting P. falciparum Translation as a Drug Target

by

Christine Moore Sheridan

DISSERTATION

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To Ben, for “hanging in there” with me

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Contribution of co-authors to the presented work

In Chapter 2 of this dissertation, Vida Ahyong performed the initial development of the PfIVT assay and buffer recipes. The construct diagram in Figure 2.3 is reproduced from Ahyong V[1], Sheridan CM[1], Leon KE[1], Witchley JN[1], Diep J[1], DeRisi JL[1,2]. "Identification of Plasmodium falciparum specific translation inhibitors from the MMV Malaria Box using a high throughput in vitro translation screen." *Malar J.* 2016.

Chapter 3 is based on a manuscript that has been submitted for publication. The following co-authors helped collect data and perform data analysis: Valentina Garcia[1] and Vida Ahyong[1]. Joseph DeRisi[1,2] supervised this work.

In Chapter 4, the ribosome profiling data in the top panel of Figure 4.1 is based on ribosome profiling data from Vida Ahyong[1] (presented in Caro F[1], Ahyong V[1], Betegon M[1], DeRisi JL[1,2]. "Genome-wide regulatory dynamics of translation in the Plasmodium falciparum asexual blood stages." *Elife*. 2014.). Alignment of ribosome profiling data to the newly identified ARE, absent from the initial work, was performed by Valentina Garcia[1]. Elfadil Osman[1] and Giselle Knudsen[3] helped collect and analyze data. Laura Jeliffe-Pawlowski[4,5] provided samples.

1 Department of Biochemistry and Biophysics, University of California, San Francisco, California.
2 Chan Zuckerberg Biohub, San Francisco, California.
3 Department of Pharmaceutical Chemistry, University of California, San Francisco, California.
4 Department of Epidemiology and Biostatistics, University of California San Francisco School of Medicine, 94107, San Francisco, CA
5 California Preterm Birth Initiative, University of California San Francisco School of Medicine, 94107, San Francisco, CA

Probing Translational Regulation by the Malaria Parasite *Plasmodium falciparum*: Applying a Novel *In Vitro* Assay to Identify Genetic Determinants of Regulation and Identify Small Molecules Exploiting *P. falciparum* Translation as a Drug Target

Christine Moore Sheridan

ABSTRACT

Over half of all pregnancies worldwide occur in malaria endemic regions. Placental malaria, a serious condition caused by the malaria parasite *Plasmodium falciparum*, occurs when malaria-infected red blood cells adhere to the tissue of the placenta, with potentially devastating consequences for both mother and infant. Placental malaria infections are responsible for approximately 30% of preventable low birth weight newborns, 20% of stillbirths, and 200,000 infant deaths per year in Africa alone. Placental malaria infection is mediated by VAR2CSA, a *P. falciparum* protein that is expressed by the parasite only when in a pregnant woman, and translationally repressed outside of pregnancy. However, the mechanisms by which this repression and expression occur or, indeed, how the parasite senses when its host is pregnant are unknown. Elucidation of the genetic determinants of this specific translational regulation could provide insight for therapeutic development for placental infection. Additionally, further study of overall translation and its pharmacologic inhibition under “normal” circumstances may help identify novel therapies for malaria in general.

Utilizing a novel *in vitro* translation system derived from *P. falciparum* cultures, I have shown that synthesis of VAR2CSA is repressed under normal conditions, and that multiple elements in the 5' untranslated region of the *var2csa* gene contribute to this repression. Further, this repression occurs only in *P. falciparum*, and not mammalian *in vitro* translation systems, indicating a *P. falciparum*-specific mechanism of inhibition. Importantly, I have found that circulating factors present in maternal serum during the first and second trimesters of pregnancy relieve repression of VAR2CSA translation, identifying two placental enzymes as candidate factors. Both enzymes serve to alter the pH of the microenvironment and, in fact, increasing pH in the *P. falciparum in vitro* translation system mimics the increase in VAR2CSA production induced by pregnant serum. Separately, I have utilized this *in vitro* translation system to identify inhibitors of translation among clinically approved antimalarial drugs and found that none utilize this mechanism of action. Importantly, this disproved the recent assertion that mefloquine inhibits translation, while also underscoring the therapeutic potential for targeting the translational apparatus as a novel and orthogonal mechanism of action.

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CHAPTER 1. INTRODUCTION

Malaria is a deadly, mosquito-borne illness, caused by a unicellular parasite, *Plasmodium.* Despite great advances made through a concerted worldwide effort in the past decades to eliminate (and eventually eradicate) the disease, malaria remains a significant global health problem. While the geographic footprint of the disease is shrinking, with 44 countries reporting fewer than 10,000 malaria cases in 2016, as compared to 37 countries in 2010, the most recent data also demonstrate an increase in total number of cases, from an estimated 211 million cases in 2015 to 216 million cases in 2016, paired with a plateau in previously declining mortality rates, with approximately 445,000 deaths in both 2015 and 2016 (1). Resistance of the mosquito vector to pyrethroid insecticides, combined with a growing mosquito habitat resulting from climate change and geopolitical instability in malaria endemic regions, particularly in the context of decreased global funding, pose significant challenges to combatting the disease (1). In particular, rising resistance of the parasite to existing antimalarial therapies highlight the importance of developing improved drugs with novel and orthogonal methods of action, and the need for a better understanding of the biology of the parasite to enable such development.

While the burden of malaria is significant for all communities and individuals residing in endemic regions, pregnant women are especially susceptible to malaria infection, particularly if experiencing their first pregnancy (2). Every year, over 125 million women become pregnant in areas of stable malaria transmission (3–5). Pregnant women who become infected with *Plasmodium falciparum* may develop

placental malaria, wherein parasites sequester on the maternal surface of the placenta, with potentially devastating consequences for both mother and the developing infant. Placental malaria results in maternal anemia, spontaneous abortion and stillbirth, premature birth, and low birth weight, even in full-term infants (2,3,6). Prematurity and low birth weight are associated with poor health outcomes and increased infant mortality, particularly in the areas in which malaria is endemic, which often have poor healthcare infrastructure. In endemic settings, placental malaria accounts for nearly one-third of all preventable low birth weight newborns, and in Sub-Saharan Africa alone, placental malaria is responsible for an estimated 200,000 yearly newborn deaths (4).

Study of protein synthesis (or translation) and its regulation within *Plasmodium* has proven to be an important, largely unexploited area of parasite biology, yielding great potential for development of antimalarial therapies. One such therapy showing great promise is the compound DDD107498, a highly specific inhibitor of *Plasmodium* translation, currently in Phase I clinical trials (7). Careful investigation of translation has historically been impeded by a lack of direct measures of translation, necessitating reliance on indirect measures prone to producing artifactual and misleading data. We have developed the *Plasmodium falciparum in vitro* translation (PfIVT) assay, allowing direct measure of parasite translation in whole-cell extracts (8). Application of the PfIVT assay has enabled us to study both general “global” translation within the parasite, probing pharmacologic inhibitors of translation as potential antimalarial therapies, as well as translational regulation of specific genes, such as *var2csa*, which is involved in placental infection. Before delving into the details of each in the ensuing chapters, a

more detailed discussion of parasite biology, and the differences in pregnant and nonpregnant hosts will be useful to lay the foundation for this work.

1.1 BIOLOGY OF THE MALARIA PARASITE

Malaria is caused by an intracellular apicomplexan parasite, *Plasmodium.* Five plasmodium species are known to infect humans (*P. falciparum, P. knowlesi, P. malariae, P. ovale, P. vivax*), with *P. falciparum* currently posing the greatest global health challenge as the most prevalent and most deadly species of malaria (1). The parasite requires two hosts to complete its life cycle: the sexual stages take place in the female *Anopheles* mosquito, and the asexual stages take place in humans (9). In the human stages, the parasites first grow and multiply in the liver (the liver stage), releasing thousands of parasites, termed merozoites, into the blood stream, which then invade erythrocytes (red blood cells or RBCs), commencing the intraerythrocytic development cycle (IDC, or blood stages), in which the parasites develop and multiply, rupturing the host erythrocyte to invade new erythrocytes and begin the cycle again (9). During the IDC, the parasite progresses through four general morphological stages of development: rings to early trophozoite to late trophozoite to schizont, before releasing new merozoites into the blood stream, rupturing the infected erythrocyte. This cycle takes place over the span of approximately 48 hours, with the synchronized rupturing of erythrocytes by many parasites producing the cyclical fevers and chills characteristic of malaria infection. In severe cases, malaria infection can result in organ failure, seizures, coma, and even death.

One particularly interesting aspect of *P. falciparum* biology, and a key to its success as a parasite throughout the ages, is its possession (and regulation) of the *var* gene family. The *P. falciparum* genome contains approximately 60 closely related *var* genes, encoding for *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a parasite protein that is exported to, and displayed on, the external surface of the erythrocyte membrane (10–12). This displayed PfEMP1 protein binds to various host receptors, glycosaminoglycans, and other moieties in the vasculature, enabling the parasite to sequester in the host peripheral circulation and avoid clearance by the spleen (10–12). Antigenic variation provided by the different *var* genes allows the parasite to evade the immune system; only one *var* gene is expressed in a population of parasites at a time, through mutually exclusive transcription (with one exception, to be discussed below), and as one variant becomes recognized by the immune system, another is expressed (10–12). Thus, the parasite utilizes its available array of PfEMP1 proteins to evade clearance by both the immune system and the spleen.

1.2 BIOLOGY OF PLACENTAL MALARIA INFECTION

The setting of pregnancy presents a unique opportunity to *P. falciparum*, in the form of a particularly beneficial niche, and it has adapted the *var* gene biology to take advantage of this opportunity. During pregnancy, a temporary organ, the placenta, rapidly develops, establishing a physical connection between the mother and the developing fetus, supporting its growth and development (13,14). Several aspects of placental biology make it favorable as a niche for the parasite. The placenta promotes

immune tolerance of the “foreign” genetic material of the fetus, as well as the placenta itself, which invades the uterine wall and vasculature with a tumor-like phenotype, and thus it provides a relatively immune-privileged environment for the *P. falciparum* parasite (13,14). In addition, the placental blood space, which contains 150 milliliters of maternal blood at term, and exchanged 3 to 4 times per minute, is extremely rich in nutrients being delivered to the developing fetus, which are then co-opted by the infecting parasites (14). Lastly, the highly efficient transfer of oxygen from maternal to fetal circulation yields a lower oxygen tension at the maternal surface of the placenta relative to other areas of maternal circulation, which is optimal for *P. falciparum* growth. It is important to note that the infected erythrocytes (iRBCs) remain on the placenta itself, and do not infect the developing fetus (15). Even so, the resulting loss of nutrients, denudation of the placenta, and chronic low-level inflammation are all incredibly detrimental to fetal health and development (15). Perhaps most concerning, parasites are often so effectively sequestered on the placenta that the mother may show no outward signs of illness and diagnostic tests may be negative, making it less likely that the mother would receive antimalarial treatment (2,16).

The blood stage parasite utilizes one PfEMP1 variant, VAR2CSA, to sequester on the maternal surface of the placenta (17–19). To enable the parasite to take advantage of the placental niche before being recognized by the immune system, the parasite “saves” this variant, and it is therefore only expressed when the parasite is in a pregnant host. In contrast to the other *vary* genes, which are transcriptionally regulated, *var2csa* is regulated at the level of translation: *var2csa* transcripts are always expressed (as the exception to the mutually-exclusive transcription rule for *var* gene transcription),

but the protein is only present in pregnant women (17–20). Indeed, *var2csa* transcripts have been found in parasites from nonpregnant individuals (men and children) (19,21). This translational regulation likely allows a rapid response by the parasite to the setting of pregnancy, which may be particularly important because exposure of the parasite to the placental circulation may be transient and brief, and the parasite must be able to express VAR2CSA quickly. Ribosome profiling data indicate that this absence of protein is not due to protein degradation, but rather lack of protein synthesis, as the ribosomes accumulate on the 5' untranslated region (5'UTR) of the mRNA transcript, suggesting a regulatory role for the VAR2CSA 5'UTR (22).

1.3 SUMMARY OF APPROACH AND FINDINGS

An important outstanding question of placental malaria is how does the malaria parasite "know" that it is in a pregnant host? More specifically, how is the parasite keeping translation of VAR2CSA turned off under nonpregnant conditions, what is it sensing to recognize pregnancy, and what is the mechanism for turning on translation of VAR2CA? In this work, I seek to answer these questions, and describe the unique methodology that we have developed to study this biology. Additionally, I apply this methodology to investigate global translation, and the potential for utilizing small molecule inhibitors as antimalarial therapies.

In Chapter 2, I introduce the *Plasmodium falciparum in vitro* translation (PfIVT) assay, a direct measure of parasite translation through use of luciferase reporter constructs in whole-cell extracts derived from *P. falciparum* blood-stage cultures. I

describe, in great detail, the methods and particulars of the assay, including the extensive validation and optimization performed, beginning with culturing parasites for the assay. In Chapter 3, I present the rigorous characterization of the PfIVT assay performed to confirm that it does, in fact, recapitulate the translational activity *in vitro* that we know to take place *in vivo.* In so doing, I present an application for the assay (one of many) as a screen for inhibitors of *P. falciparum* translation, and demonstrate that there are no direct *P. falciparum* translation inhibitors amongst the current pantheon of clinical antimalarials. Chapter 4 discusses the work I have done studying translational regulation of VAR2CSA in placental malaria, identifying both cis-acting regulatory factors within the 5'UTR of the VAR2CSA gene itself, as well as trans-acting factors from the host microenvironment that contribute to this regulation. In Chapter 5, I discuss these findings, the ongoing work arising from them, and the important future directions and next steps that can be taken in these projects.

CHAPTER 2. VALIDATION AND OPTIMIZATION OF THE PLASMODIUM FALCIPARUM IN VITRO TRANSLATION ASSAY

2.1 INTRODUCTION

Elucidation of the process of protein synthesis, or translation, and its regulation within the malaria parasite *P. falciparum* is a critical component of understanding the biology of the parasite. Illuminating “global” changes in translation within the parasite, as well as targeted regulation of translation of specific genes either in different stages of the parasite life cycle or in particular environments (i.e. the setting of host pregnancy), provides the potential to exploit this particular biology to treat and prevent the disease. However, such study of translation has been limited by the lack of a direct method of measurement. To address this, we have developed the *P. falciparum in vitro* translation (PfIVT) assay, a robustly reproducible, scalable, and high-throughput *in vitro* method to study translation in whole-cell extracts derived from *P. falciparum* blood-stage cultures, which we have previously shown replicates, *in vitro*, the relative translational efficiencies determined via ribosome profiling in live parasites (8).

In this chapter, I describe, in detail, the complete method, beginning with proper culture of parasites specifically for the PfIVT assay, as it is the foundation of much of the data upon which later chapters rely. Throughout, I highlight the thorough validation and optimization that we perform to ensure consistent reproducibility of results and maximize dynamic range of the assay. Only those extracts surpassing a rigorous activity threshold were utilized for the PfIVT assay, and extracts from individual harvests

meeting this criterion were combined to generate large pools for use across many assays. Through meticulous attention to detail and maintaining strict thresholds for assay quality and activity, we have established an assay that can be utilized for a myriad of purposes, including drug screens for translation inhibitors, studying translation regulation of individual genes, and which we are confident faithfully recapitulates the various steps of translation *in vitro,* as will be detailed in the following chapter.

2.2 DETAILED PFIVT EXTRACT GENERATION PROTOCOL

2.2.1 Parasite Culturing and Harvest Conditions for PfIVT

Proper parasite culture conditions are particularly important in generating quality extracts. To achieve high parasitemia, we utilize the *P. falciparum* strain W2 (obtained from MR4), for its hardiness and robust reinvasion rates. In brief, for each individual harvest, 1 liter of synchronized W2 culture in 2 x 500mL HYPERFlask M Cell Culture Vessels (Corning #10031) is maintained in human erythrocytes at 2-4% hematocrit and 10-20% parasitemia, at 37°C, 5% O2, and 5% CO2, and harvested upon reaching the late trophozoite/early schizont stage prior to schizont segmentation. Untreated HYPERFlasks are utilized to reduce erythrocyte adherence to the vessel. Synchronization of cultures is important for maximizing output of the harvests, therefore cultures utilized to seed the HYPERFlasks are double-synchronized to achieve tight synchronicity, either with 2 standard sorbitol synchronizations, or 1 sorbitol and 1 MACS purification (if MACS purifying, it is especially important to be extra careful and attentive

to maintaining sterility and preventing contamination). We aim to harvest as late as possible in the parasite life cycle for two reasons: (1) this maximizes the amount of extract generated (volume of the parasites is greatest) and (2) based on ribosome profiling experiments from living cultures, parasites become more translationally active the further they progress in the asexual life cycle. However, parasites must be harvested prior to schizont segmentation, to ensure efficient lysis of parasite membranes and recovery of active ribosomes. It is important to note that our protocols are optimized for harvesting at the late trophozoite/early schizont stage: generation of PfIVT extracts from other stages is possible, but the harvest procedures will likely require adjustment and further optimization.

For optimum PfIVT performance, parasites must be healthy and actively growing at the time of harvest. Several factors contribute to health of the parasites, in particular media quality, erythrocyte health and preparation, and sterile technique.

2.2.1.1 Media Preparation and Storage

We have found that media made freshly from powdered stocks yields far superior results in terms of parasite health and ultimate performance than using pre-made liquid media. Therefore, we utilize RPMI 1640 powder with L-glutamine and phenol red (Gibco #31800-022), supplemented with 0.25% w/v Albumax II (Life Technologies #11021-045), 2g/L sodium bicarbonate, 0.1mM hypoxanthine, 25mM HEPES (pH 7.4), and 50µg/L gentamicin. Media should be properly adjusted to pH7.4, sterile-filtered (0.2µm nitrocellulose), and stored at 4°C for no more than 2 weeks, and should be pre-

warmed to 37°C prior to addition to cultures. To change media, HYPERFlasks should be mixed to resuspend settled erythrocytes (RBCs) and parasite-containing erythrocytes (iRBCs), and then carefully emptied into sterile 500mL polypropylene centrifuge tubes (Corning #431123). Tubes are centrifuged at 1500rpm in a tabletop centrifuge for five minutes at room temperature without brake. When changing media in HYPERFlasks, fresh pre-warmed replacement media should be added to the HYPERFlasks as quickly as possible while the RBCs/iRBCs are being centrifuged. HYPERFlasks containing new media should then be placed in the incubator during the remainder of the spin to allow as much time as possible for equilibration of temperature and gas exchange for the vessel. After centrifugation is complete, spent media should be carefully removed, and RBCs/iRBCs should be resuspended in fresh, pre-warmed media and returned to the HYPERFlasks. HYPERFlasks should then be vigorously mixed with rotation and some gentle shaking to ensure even distribution of RBCs/iRBCs throughout the layers of the flask. HYPERFlasks should be filled to the base of the neck with liquid to prevent drying of surfaces and reduce formation of bubbles during mixing.

2.2.1.2 Media Exchange Schedule

It is important to change media frequently not only to replenish glucose and other nutrients, but also to remove waste by-products from both parasites and host erythrocytes, and maintain the pH of the culture. Parasite health and translational rates are highly subject to appropriate pH, and buffering capacity of the media can be quickly

surpassed when growing high hematocrit and/or parasitemia cultures. Therefore, media should be changed frequently: every 8-12 hours. However, the process of media exchange also places stress upon the parasites, therefore the final media change should take place no fewer than 4 hours prior to harvest to allow parasites to properly recover.

2.2.1.3 Erythrocytes for Blood Stage Culture

Fresh erythrocytes are important for both the health and re-invasion rate of the parasites, allowing for harvest of high parasitemia cultures that are very translationally active. Ideally, leukocyte-reduced whole blood will be stored at 4^{o}C for up to 2 weeks (3 weeks if absolutely necessary). The serum and buffy coat are then removed prior to use, and the erythrocytes washed and re-suspended to 50% hematocrit in fresh, pre-warmed RPMI supplemented as described above. It is important the erythrocytes are washed and resuspended with fresh media, such that neither the blood nor the media are stored for more than 2 weeks prior to addition to HYPERFlask cultures.

2.2.1.4 Sterility

HYPERFlasks, particularly with extended use and frequent media changes, pose a unique risk for contamination, especially given the requirement for the use of the relatively weak antibiotic gentamicin in Plasmodium cultures. Thus, proper sterile technique is of the utmost importance. Use of dry baths to pre-warm media, or frequent

cleaning and sterilization of water baths can make a large difference in reducing the risk of contamination of cultures. Most importantly, be extremely careful when changing media in HYPERFlasks, and be sure to remove any liquid around the mouth of the HYPERFlask either by suctioning with a sterile pipette or carefully wiping with autoclaved paper towels. If a break in culturing is needed, used HYPERFlasks can be rinsed and refilled with sterile PBS and stored in the incubator. Each HYPERFlask can be utilized for up to 1 month, but we recommend discarding after 2 weeks of continuous culturing and media changes. If there is any loss of integrity of the exterior plastic of the HYPERFlask, discard immediately, even if there is no apparent breach to the interior.

2.2.2 Parasite Harvest & Saponin Lysis for PfIVT

In brief, parasites are harvested in the late trophozoite stage at 15-20% parasitemia by centrifugation for 5 minutes at 1500rpm in a tabletop centrifuge without brake at room temperature, followed by careful removal of media and saponin lysis to remove erythrocytes. Among the most critical factors influencing the activity of extracts is the amount of saponin utilized in the erythrocyte lysis stage of harvest; the generation of active PfIVT extracts requires a much more refined erythrocyte lysis condition than, for instance, isolation of genomic DNA or preparation of proteins for western blotting.

2.2.2.1 Preparation of Saponin

Because Saponin Quillaja (Sigma #S4521) is a natural product isolated from tree bark, preparations are very heterogeneous. Content of the active lysing agent, sapogenin, ranges from 20-35%, and considerable variation in potency exists even within lots. We therefore prepare large batches (10 grams) of saponin at 0.15% w/v in phosphate buffered saline. This preparation is subjected to dual-stage filtration: first filtered with 0.8μm nitrocellulose filtration to remove large particulate, then 0.2μm nitrocellulose sterile filtration. Filtered saponin solution is then frozen and stored in 50mL aliquots at -20°C, prior to testing/calibration.

2.2.2.2 Calibration of Saponin Preparations

Each batch is calibrated empirically in a pairwise manner with test harvests to determine the optimum amount of aliquoted saponin stock to be used (FIGURE 2.1). For calibration of saponin, parasites should be cultured at 2% hematocrit and 15-20% parasitemia. Each test harvest consists of 1 liter of culture in 2 HYPERFlasks. HYPERFlasks should be mixed to resuspend settled RBCs/iRBCs, and then carefully poured into sterile 500mL polypropylene centrifuge tubes. Tubes are centrifuged at 1500rpm in a tabletop centrifuge for 5 minutes at room temperature without brake. Media is then carefully removed from the RBCs/iRBCs. At this point, RBCs/iRBCs from both tubes is resuspended in a small amount of ice-cold Buffer A (20mM HEPES pH8.0, 2mM Mg(OAc)2, 120mM KOAc) and mixed together to create one pool of culture. This

culture is then evenly divided between 2x50mL conical-bottom tubes (tubes should be rated for high speeds above 10,000x*g*) and placed on ice. The volume in one tube (Tube 1) should be brought up to 42mL with ice-cold Buffer A, and the volume in the other tube (Tube 2) should be brought up to 40mL with ice-cold Buffer A. Ice-cold saponin, previously thawed at room temperature and then placed on ice, is added as quickly as possible to the tubes to bring the volume up to 50mL in each tube: 8mL saponin to Tube 1 and 10mL saponin to Tube 2 (FIGURE 2.1). The tubes are then quickly inverted 2-3 times (first making sure the caps are properly threaded and screwed on tightly), returned to ice, and then centrifuged immediately at 10,000x*g* at 4^{o}C for 10 minutes with low brake. Keeping tubes on ice, carefully but quickly remove supernatant, then resuspend in each pellet in 45mL ice-cold Buffer A to wash, and then repeat centrifugation at 10,000x*g*. Repeat wash and centrifugation once again. After wash, carefully remove supernatant, and resuspend each pellet in an equal volume of Buffer B2 (20mM HEPES pH 8.0, 100mM KOAc, 0.75mM $Mg(OAC)_2$, 2mM DTT, 20% glycerol, 1X cOmplete EDTA-free protease inhibitor cocktail (Roche #4693132001)). For instance, if parasite pellet after washes is 1mL in volume, add 1mL of Buffer B2 for a total of 2mL volume. Transfer the parasites resuspended in Buffer B2 to separate 1.5mL screw-top tubes, flash-freeze in liquid nitrogen, and store at -80^{o}C. Process the pellets according to the protocol detailed in Section 2.2.2.3 (Harvest and Saponin Lysis of Parasites for PfIVT), below. Then, test each in the PfIVT assay as detailed in Section 2.3.4 (Pre-pooling Initial Quality Testing of Extracts), below, across a range of added magnesium (2.0, 2.5, 3.0, 3.5mM added). Whichever saponin amount yielded the extract with the highest signal, utilize that amount in the next pairwise test, following the

flow chart in Figure 2.1, and repeating testing until the optimum saponin concentration is determined. For instance, if 10mL of saponin yields the more active extract in the test Harvest 1, 10mL and 12mL will be utilized in test Harvest 2; if 10mL of saponin yields more active extract in Harvest 2, 10mL and 9mL will be utilized in Harvest 3; and so on, according to the flowchart (FIGURE 2.1). This will require 3 to 5 rounds of harvesting and pairwise testing to determine the appropriate amount of saponin to use from each large batch of saponin solution generated. Utilize this empirically determined amount of saponin for all lyses conducted with aliquots from that batch of saponin.

2.2.2.3 Harvest and Saponin Lysis of Parasites for PfIVT

For all steps, it is important to remember that time and temperature of saponin exposure is critical to prevent over-lysis. Saponin addition, lysis, and washes must be conducted at 4°C or on ice, with all manipulations executed as quickly as possible. HYPERFlasks are vigorously mixed to resuspend settled RBCs/iRBCs, and then carefully poured into sterile 500mL polypropylene centrifuge tubes. Tubes are centrifuged at 1500rpm in a tabletop centrifuge for 5 minutes at room temperature without brake. Media is then carefully removed from the RBCs/iRBCs, and parasites are resuspended in ice-cold Buffer A and transferred to 50mL conical tubes on ice. Ice-cold 0.15% saponin in Buffer A is added to resuspended parasites, and tubes (after ensuring caps are threaded correctly and tightly sealed) are inverted 2-3 times, returned to ice, and centrifuged immediately at 10,000x*g* and 4°C for 10 minutes with low brake. Volume of Buffer A and 0.15% saponin to be utilized will vary with saponin preparation

and should be determined empirically according to procedure outlined above in Section 2.2.2.2 (Calibration of Saponin Preparations). Additionally, each 50mL of Buffer A + saponin (+ parasites) is based upon the culture conditions of 2% hematocrit in 1x500mL HYPERFlask: a separate 50mL volume is required for each 500mL HYPERFlask, and an additional 50mL volume is required for each additional 2% hematocrit per HYPERFlask. For instance, if 2x500mL HYPERFlasks are grown at 3% hematocrit, 3x50mL will be required; if 2x500mL HYPERFlasks are grown at 4% hematocrit, 4x50mL will be required.

Following centrifugation, tubes are placed on ice, and supernatant is immediately and carefully removed (the pellet may be loose following the first spin). Pellets are resuspended in 45mL ice-cold Buffer A to wash, and centrifuged immediately at 10,000x*g* and 4°C for 10 minutes with low brake. Pellets are again resuspended in ice-cold Buffer A: at this point, combine pellets from all tubes in 45mL total volume and transfer to new 50mL tube (even tubes rated for very high speeds often lose integrity or begin to develop hairline cracks or "feathering" with multiple spins). Tubes must be weighed to properly balance at this stage – the density will be different than water and balance cannot be assessed by volume alone. Tubes are again centrifuged at 10,000x*g* and 4°C for 10 minutes with low brake, followed by careful removal of supernatant. Volume of the remaining pellet is estimated, and the pellet is resuspended in an equal volume of ice-cold Buffer B2 (i.e. if there is 1mL of pellet, add 1mL of Buffer B2 and mix to resuspend). This resuspended pellet is immediately transferred to a pre-chilled screw-top tube or tube(s), flash-frozen in liquid nitrogen, and stored at -80°C until read to homogenize.

2.2.3 Homogenization and Extract Generation

Following saponin lysis to remove erythrocytes, parasites are mechanically lysed to isolate the extracts utilized in the PfIVT assay. Careful mechanical lysis is required via ball homogenizer with 4μm clearance (Isiobiotec, Germany) to yield quality extracts. Mechanical lysis is necessary as the PfIVT assay is negatively affected by detergents. Use of the ball homogenizer ensures a thorough and consistently reproducible mechanical lysis, while also likely preventing overly vigorous processing of extracts and minimizing oxidation. Interestingly, while bead-mill homogenizers yield appropriate preparations of intact ribosomes for methods such as ribosome-footprinting, the PfIVT activity of extracts generated by bead-mill is generally significantly lower and much more variable than that of extracts generated by use of the ball homogenizer.

2.2.3.1 Preparation for Homogenization

While preparing the homogenizer, aliquots of Buffer B2 are thawed on ice. 1.5mL Buffer B2 are required to equilibrate the homogenizer. If more than one PfIVT harvest is to be processed, an additional 4.5mL (3x1.5mL washes) is required to wash the homogenizer in between processing of separate harvests.

Also while preparing the homogenizer, the first PfIVT harvest to be processed is thawed on ice. A minimum of 1.5mL and a maximum of 3mL can be homogenized at one time. If a single harvest comprises more than 3mL, only those aliquots that will be homogenized at the same time are thawed. Aliquots for subsequent rounds of

homogenization, or additional harvests to be homogenized are thawed (on ice) during the homogenization immediately preceding. (For example, consider 3 separate harvests: A, B, and C. Thaw A while preparing the homogenizer. Thaw B while homogenizing A. Thaw C while homogenizing B.)

The ball homogenizer is assembled with the 4μm-clearance ball bearing and pre-chilled on ice. 4x3mL Luer lock syringes and 2x16-gauge needles are also pre-chilled on ice. (An additional 8 syringes and 1 needle will be required for each additional harvest to be homogenized). It is important that all steps during the homogenization and extract processing be carried out using standard RNA handling precautions to prevent contamination with RNase. Therefore, neither the syringes, needles, nor syringe ports of the homogenizer come into direct contact with the ice while pre-chilling or during the procedure.

To equilibrate the homogenizer, one 3mL Luer lock syringe is attached to one port. 1.5mL ice-cold Buffer B2 is drawn into another 3mL Luer lock syringe utilizing a 16-gauge needle. The needle is removed (if homogenizing more than one harvest, needle can be saved for additional Buffer B2 steps) and any air expelled from the syringe. This syringe is attached to the other homogenizer port. Buffer B2 is passed from one syringe to the other 3 times, then the syringe containing the Buffer B2 is removed (while gently drawing back on the plunger to help remove any excess liquid from the homogenizer) and the Buffer B2 is forcefully expelled, emptying the syringe. To ensure that all excess Buffer B2 is removed from the homogenizer (and thus avoid diluting the harvest during processing through addition of buffer), 3mL of air are drawn into the empty syringe, which is then attach to the homogenizer port. Tipping the

homogenizer such that the air-containing syringe is higher than the empty syringe (so that any remaining liquid can run down the homogenizer channel toward the empty syringe), the air is injected into the homogenizer. The injected air and remaining Buffer B2 are ejected into the previously empty syringe. This syringe is then removed while gently drawing back on the plunger to draw out any excess liquid. This injection of air is repeated to clear out any traces of Buffer B2, and both syringes are discarded in appropriate sharps disposal.

2.2.3.2 Homogenization of Parasites to Generate PfIVT Extracts

A new (pre-chilled) syringe is attached to one port of the homogenizer. Utilizing a new (pre-chilled) 16-gauge needle, 1.5-3.0mL thawed PfIVT harvest is drawn into another new (pre-chilled) syringe. The needle is removed and carefully discarded in an appropriate sharps disposal. Any air is carefully expelled from the syringe, and the syringe is attached to the other port of the homogenizer. Then, either by hand or (preferably) utilizing the homogenization robot described in Garcia, *et al.* 2018., the harvest is passed between syringes 20 times, on ice (23). To collect the homogenized harvest, the homogenizer is tilted such that the empty syringe is higher than the syringe containing the homogenate, so that material remaining in the homogenizer will run down the homogenizer channel. The syringe containing the homogenate is carefully removed, while gently pulling back on the plunger while detaching to draw out any additional liquid. The contents of the syringe are carefully emptied into a pre-chilled Raze-free microcentrifuge tube (or tubes) on ice. To maximize homogenate collection,

3mL air is drawn into the now empty syringe, and the syringe is then attached to the open port. Tilting the homogenizer such that the empty syringe is higher than the air-filled syringe, the air is forcefully injected into the homogenizer. The air and remaining homogenate should pass into the empty syringe. The syringe containing the homogenate is carefully removed, gently pulling back on the plunger while detaching to draw out any additional liquid. The contents of the syringe are carefully added to the microcentrifuge tube(s). This injection of air is repeated once more, collecting any remaining homogenate. Collected homogenate is then centrifuged as outlined below in Section 2.2.3.3 (Extract Collection).

If more volume from the same harvest remains to be homogenized (i.e. if there were more than 3mL of the same harvest), this entire process is repeated with the remaining volume of the harvest while centrifuging the first volume.

2.2.3.3 Extract Collection

Tubes containing homogenate are immediately centrifuged at 16,000x*g* and 4°C for 10 minutes. Supernatant is carefully transferred to a pre-chilled RNase-free 2.0mL microcentrifuge tube or tubes. 100μL is transferred to a separate pre-chilled RNase-free 0.5mL microcentrifuge tube. This aliquot will be utilized for extract quality testing. If there are more than 3mL volume of a single parasite harvest, requiring more than one round of homogenization & subsequent extract collection, all collected extracts from the same harvest are combined (gently mixing by pipetting) prior to removing the aliquot for quality control (i.e. If there are 4mL of harvest, resulting in 2 homogenization batches of

2mL each, the collected extract from both homogenization batches is mixed in one tube, then the 100μL aliquot for extract quality testing is removed from this combined batch).

All tubes are flash-frozen in liquid nitrogen and stored at -80°C until ready to proceed with quality control testing. Even if proceeding to quality testing of the 100μL aliquot immediately, the aliquot must first be flash-frozen. Extract activity is lost with each freeze/thaw cycle, and it is imperative that the test aliquot is representative of the harvest from which it is derived; if the aliquot were not first frozen, it would have greater activity than the rest of the harvest.

2.2.3.4 Homogenizer Cleaning

If multiple harvests are to be homogenized, the homogenizer is washed 3 times with 1.5mL Buffer B2 in between processing different harvests, using a new pre-chilled pair of syringes for each wash. For each wash: one 3mL Luer lock syringe is attached to one port. 1.5mL ice-cold Buffer B2 is drawn into another 3mL Luer lock syringe using the 16-gauge needle saved from the Buffer B2 equilibration step. The needle is removed (saving for additional Buffer B2 steps), and any air expelled from the syringe. This syringe is attached to the other homogenizer port. Buffer B2 is passed from one syringe to the other 3 times, then the syringe containing the Buffer B2 is removed (while gently drawing back on the plunger to help remove any excess liquid from the homogenizer) and Buffer B2 is forcefully expelled, emptying the syringe. To ensure that all excess Buffer B2 is removed from the homogenizer (and thus avoid diluting the harvest during processing through addition of buffer), 3mL of air is drawn into the empty

syringe, which is then attached to the open homogenizer port. The air is injected, tipping the homogenizer such that the air-containing syringe is higher than the empty syringe (so that any remaining liquid can run down the homogenizer channel toward the empty syringe). The injected air and remaining Buffer B2 should be ejected into the previously empty syringe. This syringe is then removed, gently drawing back on the plunger to draw out any excess liquid. The remaining syringe is removed, and both are discarded carefully in appropriate sharps disposal. This process is repeated for a total of 3 washes. On the third wash, 2 air injection steps are performed to ensure that as much buffer as possible is removed from the homogenizer.

When all harvests have been homogenized, the homogenizer is carefully disassembled, with care taken not to scratch or otherwise damage the ball bearing. All components are thoroughly rinsed with MilliQ water to remove any remaining homogenate, then submerged in 70% MeOH for 15-20 minutes. Following MeOH soak, all components are washed thoroughly 3 times with MilliQ water to remove all traces of MeOH, and are then allowed to air dry completely.

2.3 PFIVT EXTRACT QUALITY CONTROL TESTING AND POOLING

Even with the most meticulous adherence to carefully optimized culturing, harvesting, and processing protocols, the resulting extracts naturally exhibit a range of translational activities, and only those that surpass a rigorous activity threshold are pooled and subjected to further quality control measures to yield the final extract utilized in the PfIVT assay (FIGURE 2.2). Briefly, each individually harvested extract is tested

for translational activity across a range of magnesium concentrations, with a 2-hour incubation at 37°C to detect the signal at (or near) saturation. Extracts failing to reach the minimum activity threshold of 10^4 relative luminescence units (RLU) in at least one condition utilizing a control firefly luciferase reporter are discarded. All extracts exceeding the threshold are pooled and carefully mixed, then aliquoted in 200μL volumes, flash-frozen in liquid nitrogen, and stored at -80°C. Representative aliquots from the pool are then re-tested to determine optimum magnesium concentration and incubation time for all future PfIVT assays utilizing this pool of extract (FIGURE 2.2).

For all PfIVT reactions, it is imperative to utilize RNase-free buffers and reagents, and to utilize appropriate RNA handling precautions to prevent contamination and degradation of reactions with RNase.

2.3.1 mRNA for PfIVT Quality Testing and Control Reactions

All quality control testing is performed with a firefly luciferase reporter preceded by the 5' untranslated region (5'UTR) of the *P. falciparum* EBA-175 gene, and followed by the 3'untranslated region (3'UTR) of the *P. falciparum* HRP gene (FIGURE 2.3). This construct is denoted EBA175_FLUC. All mRNA reporter constructs utilized in the PfIVT assays are transcribed *in vitro* by T7 polymerase. For the assays described in this work, the reporter constructs do not have 5'caps or polyA tails. Because these are T7-transcribed mRNA transcripts, generation of un-capped, non-polyA transcripts is both more time and cost efficient. It also generates more uniform pools of transcripts than either incorporation of cap during T7 transcription, or addition of cap and polyA tail

via post-transcriptional enzyme activity. Additionally, it has been shown in other *in vitro* translation systems, and we have demonstrated for the PfIVT system, that *in vitro* translation is not cap-dependent, and presence of cap and polyA only increases translation efficiency by a very small amount, if at all (FIGURE 2.4).

In addition to the optimization and quality control assays, all drug screens and translation inhibition assays described herein are also performed with the EBA175_FLUC mRNA in all conditions. Importantly, EBA175_FLUC mRNA without drug treatment serves as the internal control for all PfIVT assays, regardless of type of assay. Signal of the assay is considered to be acceptable if the EBA175_FLUC mRNA untreated control achieves at least 10^4 RLU. If comparing different mRNA transcripts (i.e. for studying different UTRs), mRNA is electroeluted to ensure homogeneity of transcript preparations. All mRNA constructs being compared in the same experiments are normalized at the same time to 1pmol/μL utilizing Qubit RNA HS reagents (ThermoFisher), then aliquoted, flash-frozen, and stored at -80°C. mRNA constructs are assessed via BioAnalyzer mRNA Pico chip (Agilent) to verify purity, size, and concentration of aliquoted transcript stocks.

2.3.2 Making 10X Translation Mixes for Quality Testing

Ribosome activity is greatly influenced by concentrations of metal cations and magnesium in particular (24). To determine the ideal amount of magnesium to be utilized with each pool of PfIVT extract, it is assayed across a range of added magnesium concentrations (supplied in the form of $Mg(OAc)_2$).

First, a series of $Mg(OAc)_2$ stocks are prepared, by diluting 1M $Mg(OAc)_2$ with DEPC H_2O: 150, 200, 250, 300, 350, 400, and 450mM $Mg(OAc)_2$. Next, a large master mix of 700μL of 10X Translation Mix (10X TM) without $Mg(OAc)_2$ is made (TABLE 2.1). The Translation Mix and any reactions containing it must never be vortexed! Instead, it is always mixed by gentle pipetting. The creatine kinase in the Translation Mix is very sensitive to hydrolysis, and vortexing can lead to a drastic reduction, or even complete loss, of activity in the PfIVT assay. This master mix is split into 7x90μL aliquots (there should be some master mix leftover), to which add 10μL of the appropriate $Mg(OAc)_2$ are added to yield 10X Translation Mix of 15, 20, 25, 30, 35, 40, and 45mM $Mg(OAc)_2$. When added 1:10 to the final PfIVT reactions (for a final concentration of 1X TM), these will yield added magnesium concentrations of 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5mM, respectively. 10X Translation Mixes are aliquoted into PCR strips, with each strip containing one aliquot of each magnesium concentration for easy use. Once an aliquot has been thawed, any unused 10X Translation Mix remaining should be discarded. It is very important that the 10X Translation Mixes utilized for the quality control testing be uniform in composition (excepting magnesium concentration) and handling, including freeze/thaw conditions. This ensures that the only variable being changed is the magnesium concentration, and it can be certain that any differences in PfIVT signal are due to magnesium concentration and nothing else.

2.3.3 Preparing Assay Plates to Stop PfIVT Reactions and Measure Luciferase Activity

A translation reaction stop solution of 50μM cycloheximide (CHX STOP) is prepared by diluting 1M cycloheximide in MilliQ H_2O. Great caution must be used when handling cycloheximide – it is a highly potent inhibitor of eukaryotic translation and is very toxic. Proper PPE, including eye protection, is essential. Cycloheximide and all reagents (tips, plates, etc.) that are contaminated with cycloheximide must be properly disposed of. This CHX STOP solution can be aliquoted and stored at -20°C. Using the LabCyte ECHO acoustic liquid handler, 2μL of CHX STOP are dispensed to all inner wells of white flat-bottom 96-well assay plates. The outer wells (rows A & H and columns 1 & 12) are not be used. After dispensing of CHX STOP, plates are sealed immediately with plate seals appropriate for frozen storage, and then stored at -20°C. Plates are thawed and brought to room temperature immediately prior to use. After thawing, but before removing seals, plates are briefly centrifuged to ensure that all liquid is collected on the bottom of the wells. It is very important that plates be brought to room temperature prior to luciferase readings, as the luciferase signal is heavily impacted by temperature.

2.3.4 Pre-pooling Initial Quality Testing of Extracts

To determine whether processed extracts meet the activity threshold, PfIVT reactions are performed with each extract, using the 10X Translation Mixes (15, 20, 25,

30, 35, 40, and 45mM $Mg(OAc)_2$) made in Section 2.3.2 (Making 10X Translation Mixes for Initial Quality Testing), above, and allowed to run to completion to determine the saturation signal for each extract. Because these reactions are performed only to identify which extracts meet the activity standards for pooling, and magnesium concentrations for experimental assays will need to be determined for the pool, only individual reactions are performed for each magnesium concentration; reactions need not be performed in duplicate at this stage.

2.3.4.1 PfIVT Master Mix for Initial Extract Quality Testing

The components for the PfIVT Master Mix, listed in Table 2.2, are combined in the proportions indicated in the table, on ice, and mixed by gently pipetting (NEVER VORTEX!) (TABLE 2.2). The mix is quickly spun down following mixing to eliminate air bubbles and collect liquid from walls of the tube.

2.3.4.2 Magnesium Master Mix for Initial Extract Testing

Next, combine the components for the Magnesium Master Mix, listed in Table 2.3, in the proportions indicated, on ice (TABLE 2.3). Again, the Master Mix is pipetted gently to combine (DO NOT VORTEX!) and quickly spun down following mixing. One Magnesium Master Mix is made for each concentration of magnesium. Volume is adjusted based on the number of extracts to be tested.

2.3.4.3 PfIVT Reactions for Initial Extract Testing

While the various master mixes for these quality-testing reactions are being prepared, the 100μL test aliquots (generated in Section 2.2.3.3 (Extract Collection)) for each extract are thawed on ice. 6μL of each Magnesium Master Mix (as described above in Section 2.3.4.2 (Magnesium Master Mix for Initial Extract Testing)) is dispensed to the bottom of appropriate wells of a PCR plate on ice. Then, 14μL of the thawed PfIVT extract is carefully dispensed to the sides of appropriate wells of the same PCR plate. Outer wells (rows A & H, columns 1 & 12) of plate are not utilized for PfIVT reactions. The plate is briefly centrifuged (after covering with an adhesive seal) to combine extracts and Master Mixes at the same time. Contents of all wells are mixed by gently pipetting, and again covered with an adhesive seal and briefly centrifuged. Empty wells are filled with DEPC H_2O, and the plate is firmly covered with an adhesive seal, and transferred to a 37°C incubator. Plates are incubated at 37°C for 2 hours, such that the reactions are allowed to run to (or near) saturation.

During this time, CHX STOP plate(s) (as described in Section 2.3.3 (Preparing Assay Plates to Stop PfIVT Reactions and Measure Luciferase Activity)) are thawed, and allowed to equilibrate to room temperature in the dark. The appropriate volume of firefly luciferin reagent (as described in Section 2.4.2 (PfIVT Assay: Step-By-Step Protocol)) is also thawed and allowed to equilibrate to room temperature in the dark – it is very important to protect firefly luciferin reagent from light. Unlike the CHX STOP plates, firefly luciferin reagent can be thawed at 37°C if necessary, but it must be allowed to cool to room temperature prior to use. Once thawed, the firefly luciferin

reagent is thoroughly vortexed, as some precipitate may have formed during freezing. If multiple aliquots of firefly luciferin reagent are necessary, all aliquots are combined and mixed, and this mix is thoroughly vortexed immediately prior to use in the luciferase assay. As a reminder, all reagents utilized in luciferase reactions must be at room temperature at time of use, as luciferase reactions are strongly affected by reaction temperature.

If pooling of satisfactory extracts on the same day as the test assay is performed, excess volume from the 100μL test extract can be stored at 4°C on ice until pooling. If pooling will take place on another day, excess extract is flash-frozen in liquid nitrogen and store at -80°C.

After 2-hour incubation of PfIVT reactions is completed, PfIVT plates are placed on ice. Plates are then centrifuged for 2 minutes at 2500rpm and 4°C in a tabletop centrifuge to spin down any evaporated liquid from the top and walls of wells, and again placed on ice. CHX STOP plates are briefly centrifuged at room temperature to ensure that all liquid is collected at bottom of wells (CHX STOP plates must be equilibrated to room temperature prior to luciferase assay). 17.5μL of PfIVT reactions are carefully transferred from the PfIVT reaction plate to the walls of corresponding wells of the CHX STOP plate. The CHX STOP plate, now containing the transferred PfIVT reaction, is briefly centrifuged so that all PfIVT reactions are combined with, and stopped by, CHX STOP at the same time. The plate is then read with an injection luminometer, with 200μL firefly luciferase injection volume, 200μL/sec injection speed, 3-second delay, and 3-second integration.

2.3.5 Pooling PfIVT Extracts

Extracts that achieve a minimum value of 10^4 relative luciferase units (RLU) in the initial quality testing in at least one magnesium condition are considered to have met the quality threshold for use in the PfIVT assay. Extracts failing to achieve the minimum value of 10^4 RLU are discarded.

For all satisfactory extracts (those achieving a minimum signal of 10^4 RLU), all aliquots are thawed on ice. All tubes are briefly centrifuged once thawed. All extracts are carefully transferred to the same RNase-free 50mL conical tube, including any remaining from the 100μL test aliquots. Extracts are viscous, so pipetting must be performed slowly and carefully to minimize loss. All tubes (now mostly empty) are briefly centrifuged once more, and any remaining liquid is transferred to the pool. The 50mL conical tube is briefly centrifuged to remove liquid from the sides of the tube. The pool is then gently mixed by pipetting up and down, and stirring with a 1000μL pipette. After being briefly centrifuged once more, 2x300μL aliquots of the pooled extract are carefully dispensed, and then the remainder is dispensed in 200μL aliquots, all in RNase-free 0.5mL microcentrifuge tubes. These tubes are flash-frozen in liquid nitrogen, and stored at -80°C. Again, even if proceeding immediately to quality control testing of the pooled extracts, all aliquots must first be frozen to ensure uniformity of extracts. Aliquots are then thawed to use for testing.

2.3.6 Magnesium Testing of Pooled PfIVT Extracts

Because ribosome activity is especially sensitive to magnesium, we have measured the magnesium concentrations of several PfIVT extracts, and then determine the optimal amount of magnesium required by each extract for maximal activity. Basal magnesium concentrations were typically less than 2mM, whereas maximum translational activity was achieved at a final PfIVT reaction concentration of approximately 4mM magnesium (FIGURE 2.5A&B). Having established the basal range of magnesium seen in the PfIVT extracts (see Section 2.5 (Additional Method: Magnesium Concentration Assay) for protocol), we now determine the amount of magnesium to add to reactions empirically, as described below, in lieu of measuring basal levels.

A 300μL test aliquot of pooled extract (as described above in Section 2.3.5 (Pooling PfIVT Extracts)) and a fresh strip of aliquots of the 10X Translation Mixes (as described in Section 2.3.2 (Making 10X Translation Mixes for Quality Testing)) are thawed on ice. Pooled extracts are tested in duplicate at reaction concentrations of 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5mM added magnesium.

2.3.6.1 Pool Master Mix for Magnesium Testing of Pooled PfIVT Extracts

To make the Pool Master Mix for these reactions, the components in the proportions noted in Table 2.4 are combined on ice (TABLE 2.4). This Master Mix is

pipetted gently to combine (DO NOT VORTEX!) and quickly spun down following mixing to eliminate air bubbles and collect liquid from walls of tube.

2.3.6.2 Magnesium Master Mix for Magnesium Testing of Pooled PfIVT Extracts

To prepare the Magnesium Master Mix for magnesium testing of the pooled extracts, the components noted in Table 2.5 are combined in the indicated proportions on ice (TABLE 2.5). Again, components are pipetted gently to combine (DO NOT VORTEX!) and quickly centrifuged following mixing. A separate Magnesium Master Mix is prepared for each concentration of magnesium to be tested.

2.3.6.3 Magnesium Testing Reaction for Pooled PfIVT Extracts

4μL of each Magnesium Master Mix (as described above in Section 2.3.6.2 (Magnesium Master Mix for Magnesium Testing of Pooled PfIVT Extracts)) are dispensed, in duplicate, to the bottom of appropriate wells of a PCR plate on ice. Next, 16μL of Pool Master Mix (as described above in Section 2.3.6.1(Pool Master Mix for Magnesium Testing of Pooled PfIVT Extracts)) is carefully dispensed to the wall of appropriate wells of the same PCR plate. Outer wells (rows A & H, columns 1 & 12) of the plate are not utilized for PfIVT reactions – these are instead filled with DEPC H_2O. The plate is briefly centrifuged (covered with adhesive seal) to combine reaction components at the same time in all wells. The contents of all wells of the plate are then

gently pipetted to mix, and again firmly covered with an adhesive seal, briefly centrifuged, and transferred to a 37°C incubator. The plate is incubated at 37°C for only 60 minutes. This incubation period is shorter than for the initial extract quality testing outlined in Section 2.3.4.3 (PfIVT Reactions for Initial Extract Testing), because, in this instance, the goal is not to achieve saturation to determine maximum possible output for the extract, but rather to determine which magnesium concentration yields the greatest activity for the pool. For this reason, reactions should be stopped well before reaching saturation to ensure that differences between magnesium concentrations can be detected.

During the PfIVT reaction incubation period, CHX STOP plate(s) (as described in Section 2.3.3 (Preparing Assay Plates to Stop PfIVT Reactions and Measure Luciferase Activity)) are thawed and allowed to equilibrate to room temperature in the dark. The appropriate volume of firefly luciferin reagent (as described in Section 2.4.2 (PfIVT Assay: Step-By-Step Protocol)) is also thawed and allowed to equilibrate to room temperature in the dark. Again, all reagents utilized in luciferase reactions must be at room temperature at time of use.

If planning to test kinetics of the pooled extract on the same day as the magnesium test is performed, excess extract from the 100μL test extract can be stored at 4°C on ice until pooling. Otherwise, excess extract is discarded. (Note: if conserving extract volume is an issue, or if extracts are particularly robust, thawed extract aliquots can be snap-frozen in liquid nitrogen and stored at -80°C, but it is important to document and monitor the numbers of freeze/thaw cycles of each aliquot, and

empirically determine for each pool of extracts how many freeze/thaw cycles can be tolerated by an extract while still maintaining minimum signal of 10^4 RLU.)

After the 60-minute incubation of the PfIVT reactions is completed, PfIVT plates are placed on ice. Plates are then centrifuged for 2 minutes at 2500rpm and 4°C in a tabletop centrifuge to spin down any evaporated liquid from the top and walls of wells, and again placed on ice. Thawed CHX STOP plates are briefly centrifuged at room temperature to ensure that all liquid is collected at bottom of wells (CHX STOP plates must be equilibrated to room temperature prior to luciferase assay). 17.5μL of the PfIVT reactions are carefully transferred from the PfIVT reaction plate to the walls of the corresponding wells of the CHX STOP plate. The plate is then briefly centrifuged so that all PfIVT reactions are combined with, and stopped by, CHX STOP at the same time. The plate is then read using an injection luminometer, with 200μL firefly luciferase injection volume, 200μL/second injection speed, 3-second delay, and 3-second integration.

The results for replicates from the same magnesium concentration are averaged together to determine which magnesium concentration yields the highest signal. This is the magnesium concentration that will be utilized for all future PfIVT assays using this pool of extract.

A large enough batch is made of 10X Translation Mix (see Table 2.1) at this calculated concentration of magnesium to be utilized for all assays with the remainder of the pool this pool of extract, taking care to make at least 20% extra volume (TABLE 2.1). This 10X Translation Mix is aliquoted in RNase-free tubes, flash-frozen in liquid nitrogen, and stored at -80°C. If volumes are known/estimated for planned assays

using this pool (for instance, if a drug screen is planned for a known number of compounds), 10X TM is frozen in appropriately sized aliquots for each assay. Otherwise, 10X TM is frozen in small aliquots that can be pooled as needed. Once an aliquot has been thawed, it is discarded.

2.3.7 Kinetic Testing of Pooled PfIVT Extracts

Upon determining optimal magnesium conditions for each pool of extract, kinetic curves are generated in 15-minute increments to establish the ideal incubation time for the assay (FIGURE 2.5C). Kinetics of the assay varies between extracts, and separate kinetic curves must also be established for the particular reporter utilized (for example, nanoluciferase) (FIGURE 2.5C&D). Incubation times for future assays performed with this extract pool are determined based upon the kinetic curve generated for the pool, keeping in mind the experimental goal of the PfIVT assay being performed. To maintain maximal sensitivity to inhibitors and linearity of the assay, we conducted PfIVT inhibition experiments at the time point corresponding to 75-80% of the saturation signal (FIGURE 2.5C). In contrast, for assays seeking to identify agonists of translation, or in which both increases and decreases in translation might be observed (for instance, when comparing different 5'UTRs), an incubation time corresponding to 50% of the maximum signal from the kinetic assay would yield a larger and more optimal dynamic range.

2.3.7.1 Protocol for Kinetic Testing

One of the 300μL test aliquots (as described above in Section 2.3.5 (Pooling PfIVT Extracts)), as well as the necessary aliquot(s) of the 10X Translation Mix with the appropriate magnesium concentration for the pool (as determined in Section 2.3.6.3 (Magnesium Testing Reaction for Pooled PfIVT Extracts)) are thawed on ice. The appropriate number of aliquots of firefly luciferin reagent (as described in Section 2.4.2 (PfIVT Assay: Step-By-Step Protocol)) are also thawed, allowing the reagent to equilibrate to room temperature in the dark. An aliquot of CHX STOP solution is also thawed, protecting from light (Note: here, an aliquot of CHX STOP solution, rather than a plate, is thawed because the CHX STOP solution will be added to individual reactions to stop translation at the appropriate time points).

The pooled extract is assayed in duplicate at the following time points: 15, 30, 45, 60, 75, 90, 105, 120, and 135 minutes. Kinetic testing is best performed in PCR tubes, rather than PCR plates, to allow for easy collection of time points. If using PCR strips, be sure to separate tubes into pairs prior to addition of reagent to facilitate removal of the appropriate tubes from the incubator without disturbing other reactions at time point collection.

Reaction components are combined on ice, according to Table 2.6. This reaction mix is pipetted gently to combine (DO NOT VORTEX!) and quickly centrifuged following mixing to eliminate air bubbles and collect liquid from walls of tube.

20μL of the PfIVT Kinetic Master Mix is carefully pipetted into each of 16 PCR tubes on ice. The tubes are briefly centrifuged, then placed in the incubator at 37°C. At

each 15-minute interval following start of incubation, a pair of tubes is removed and placed on ice. 2.2μL of CHX STOP solution is promptly (and carefully) added to each tube, using appropriate cycloheximide handling and disposal procedures. Making sure that the PCR tubes are securely closed, tubes are flicked to mix, then briefly centrifuged, and placed on ice. This procedure is repeated every 15 minutes with a different pair of tubes, until 150 minutes has passed and the last time point has been collected. All PCR tubes, now on ice and containing CHX STOP solution, are centrifuged once more. 18.5μL is carefully transferred from each tube to separate wells of a white-walled, flat bottom, 96-well assay plate. Liquid should be pipetted directly onto the flat bottom of the well. The plate is then read using an injection luminometer, with 200μL firefly luciferin reagent injection volume, 200μL/sec injection speed, 3-second delay, and 3-second integration.

The average of the replicates for each time point are plotted to determine the incubation time for future assays performed with this extract pool.

2.4 BUFFER RECIPES AND STEP-BY-STEP PROTOCOLS FOR PFIVT ASSAY EXTRACT QUALITY CONTROL

Here, I present simplified step-by-step protocols for the procedures described above, for easy reference in the laboratory.

2.4.1 Culturing and Extract Generation: Step-By-Step Protocol

Buffer A: 20mM HEPES pH8.0

2mM $Mg(OAc)_2$

120mM KOAc

Saponin: 0.15% w/v Saponin Quillaja (Sigma #S4521) in Buffer A

1) Double-synchronize parasites with either a) two sorbitol synchronizations or b) 1 sorbitol synchronization and 1 MACS purification
2) Culture 2x500mL HYPERFlasks of double-synchronized parasites in supplemented RPMI and 2-4% hematocrit at 37°C, 5% O_2, and 5% CO_2.
3) Change media every 8-12 hours. The final media change prior to harvest should take place no more than 8, and no fewer than 4 hours preceding the harvest.
4) Harvest parasites when they have grown to 10-20% parasitemia, and are at the late trophozoite/early schizont stage: as late as possible in the life cycle prior to schizont segmentation.
5) Harvest by centrifuging at 1500rpm for 5 minutes at room temperature in tabletop centrifuge without brake.
6) Carefully remove media.
7) Resuspend pellet in ice-cold Buffer A, transfer to 50mL conical tube on ice
    a. Volume of Buffer A will vary depending on batch of saponin utilized; to be determined empirically as described in Section 2.2.2.2 (Calibration of Saponin Preparations)

8) Add ice-cold 0.15% saponin as quickly as possible, invert 2-3 times, return tubes to ice
    a. Volume of saponin to be added will vary depending on batch of saponin utilized; to be determined empirically described in Section 2.2.2.2 (Calibration of Saponin Preparations)
9) Immediately centrifuge tubes at 10,000x*g* and 4°C for 10 minutes with low brake.
10) Keeping tubes on ice, carefully remove supernatant as quickly as possible.
11) Resuspend pellet in 45mL ice-cold Buffer A to wash.
12) Immediately centrifuge tubes at 10,000x*g* and 4°C for 10 minutes with low brake.
13) Keeping tubes on ice, carefully remove supernatant as quickly as possible.
14) Repeat wash: resuspend pellet in 45mL ice-cold Buffer A.
15) Immediately centrifuge tubes at 10,000x*g* and 4°C for 10 minutes with low brake.
16) On ice, carefully remove supernatant.
17) Estimate pellet volume, and resuspend pellet in an equal volume of Buffer B2, i.e. if pellet volume is 1mL, resuspend in 1mL Buffer B2
18) Transfer resuspended pellet to 1.5mL screw-top tube, flash-freeze in liquid nitrogen
19) Store frozen pellets at -80°C until ready to homogenize.
20) Thaw pellet(s) on ice.
21) While thawing pellet(s), prepare homogenizer: assemble with 4μm-clearance ball bearing, pre-chill homogenizer on ice, flush homogenizer with 1.5mL Buffer B2, and remove all buffer. (Ball homogenizer from Isiobiotec).

22) Draw 1.5-3mL of thawed pellet into 3mL Luer lock syringe (pre-chilled on ice) and attach to prepared homogenizer.

23) Utilizing homogenizer robot, homogenize the thawed pellet by passing from syringe to syringe 20 times, generating lysate.

    a. If homogenizing pellets from more than one harvest, wash the homogenizer in between processing separate harvests with ice-cold Buffer B2.

24) Transfer homogenized lysate to pre-chilled 1.5mL microcentrifuge tube(s).

25) Centrifuge immediately at 16,000x*g* and 4°C for 10 minutes in tabletop microcentrifuge.

26) Carefully transfer supernatant (the PfIVT extract) to fresh pre-chilled tube, setting aside 100μL of extract in a separate tube for quality control testing.

27) Flash-freeze all tubes in liquid nitrogen, store at -80°C.

28) For each harvest, determine whether extract is sufficiently active for use (see Figure 2.2 for flowchart):

    a. Thaw test aliquot, test in PfIVT assay (see Section 2.4.2 (PfIVT Assay: Step-By-Step Protocol)) with 2.0, 2.5, 3.0, 3.5, and 4.0 mM added magnesium (added in 10XTM), and 2-hour incubation time.

    b. Extracts achieving activity greater than or equal to 10^4 RLU with firefly luciferase are considered good; those with lower activity are discarded.

29) Pool all extracts that surpass the activity threshold in a 50mL conical tube on ice. Aliquot into 2x300μL test aliquots and the remainder of the pooled extract into 200μL aliquots in pre-chilled tubes, flash-freeze in liquid nitrogen.

30) Thaw two aliquots for activity testing (see Figure 2.2 for flowchart):

a. Test in PfIVT assay (see Section 2.4.2 (PfIVT Assay: Step-By-Step Protocol)) with 2.0, 2.5, 3.0, 3.5, and 4.0 mM added magnesium (added in 10XTM), and 90-minute incubation time. This should be done in duplicate. The magnesium concentration that yields the highest signal is the concentration that will be used for future assays with this pool of extract.
b. Using the appropriate magnesium concentration as determined in step a, test in PfIVT assay to determine kinetics of the extract pool. Remove duplicate samples from 37C incubation every 15 minutes until the final time point of 150 minutes, placing on ice and adding 2μL of 50μM CHX STOP (see Section 2.4.2 (PfIVT Assay: Step-By-Step Protocol)) to each sample immediately after removing from the incubator. For inhibitor screens, the incubation time used should be 70-80% of the activity at saturation, to maximize the assay window within the linear range. For different kinds of PfIVT assays, a shorter incubation may provide a better dynamic range.

2.4.2 PfIVT Assay: Step-By-Step Protocol

PfIVT ASSAY STEP-BY-STEP PROTOCOL

All reagents should be RNase-free. Proper RNA handling procedures must be followed to avoid introduction of RNase and subsequent degradation of mRNA transcripts and skewing of results.

SOLUTIONS:

10X Translation Mix

DO NOT VORTEX 10XTM or creatine phosphokinase

Aliquot, flash-freeze in liquid nitrogen, store at -80°C

20mM HEPES pH7.4

75mM KOAc

2mM DTT

5mM ATP

1mM GTP

200mM phosphocreatine

2μg/μL creatine phosphokinase

Appropriate 10X $Mg(OAc)_2$ as determined for extract pool

Firefly luciferin reagent

*Aliquot, flash-freeze in liquid nitrogen, store at -80°C *in the dark**

Per 100mL:

89.98mL ddH_2O

0.20mL EDTA (50mM)

1.66mL $MgSO_4$ (100mM)

3.33mL DTT (1M)

0.27mL CoA (100mM)

0.53mL ATP (100mM)

2mL tricine pH8.15 (1M)

1mL D-luciferin (100mM)

0.5mL NaOH (1M)

0.53mL magnesium carbonate hydroxide (50mM)

Cycloheximide stop solution (CHX STOP)

*Aliquot, flash-freeze on dry ice, and store at -20°C *in the dark**

Dilute 1M cycloheximide stock solution to 50μM in ddH_2O

1) Combine the following in PCR plates:

14μL PfIVT extract

2μL 10X Translation Mix with appropriate [Mg++]

1μL amino acid mixture (100μM)

1μL T7-transcribed firefly luciferase mRNA (1μg/μL stock)

2μL DEPC H_2O

20μL total reaction volume

* For multiple reactions, create appropriate PfIVT reaction master mix and dispense to PCR plates

** If testing inhibitors or other drugs in the assay, pre-dispense to drug to PCR-plate and spin down. Then add PfIVT reaction to walls of PCR-plate wells and spin down such that all PfIVT reactions are mixed with drug at the same time.

*** Similarly, if testing magnesium concentrations, pre-dispense $Mg(OAc)_2$ and DEPC H_2O to PCR plates. Make magnesium-free PfIVT reaction master

mix and dispense to walls of PCR-plate wells and spin down such that all PfIVT reactions are mixed with magnesium at the same time.

2) Pipette up and down gently to mix (DO NOT VORTEX)
3) If using PCR plates: fill empty wells with 20μL DEPC H_2O and cover with adhesive plate seal to prevent evaporation
4) Spin down tubes/plates briefly
5) Incubate at 37°C for appropriate amount of time
6) Spin down tubes/plates briefly, place on ice
7) Dispense 2μL CHX STOP to appropriate wells of white 96-well flat-bottom assay plate, briefly spin down to ensure no liquid on walls of plate
    a. CHX STOP plates can be prepared in advance and stored (with appropriate plate seals) for a limited time at -20°C, then thawed & brought to room temperature when needed.
    b. After thawing, briefly spin down plates before use.
    c. It is important that plates be brought to room temperature prior to addition of luciferin reagent, as luciferase enzyme activity and luminescence signal are temperature sensitive.
8) Carefully transfer 17.5μL of each PfIVT reaction to walls of wells containing CHX STOP (prepared in Step 7)
9) Spin down plates so that all PfIVT reactions mix with, and are stopped by, the CHX STOP at the same time.
10) Read plates at room temperature on injection luminometer with the following settings:

- 200μL injection of firefly luciferin reagent
- 200μL/sec injection rate
- 3-second delay
- 3-second integration

2.5 ADDITIONAL METHOD: MAGNESIUM CONCENTRATION ASSAY

Baseline magnesium levels of the PfIVT extracts (noted in Section 2.3.6 (Magnesium Testing of Pooled PfIVT Extracts)) were measured using a magnesium-dependent enzyme-based colorimetric assay kit (Sigma-Aldrich #MAK026). Two biological replicates of a dilution series of each extract were tested in duplicate with each of two separate kits, following the protocol provided with the kit. In brief, 10μL of each PfIVT extract (neat, or diluted 1:4 or 1:10 with ddH_2O) added to 10μL ddH_2O, along with a standard curve, was combined with 50μL of master reaction mix (35μL magnesium assay buffer, 10μL developer, 5μL magnesium enzyme mix), and incubated for 10 minutes with shaking at 37°C. 450nm absorbance was read immediately after the initial incubation, and every 5 minutes thereafter on a Tecan plate reader until the highest A450 approached (but did not exceed) 1.5X the initial reading. Values were fitted to, and interpolated from, the standard curve using Prism GraphPad.

2.6 FIGURES

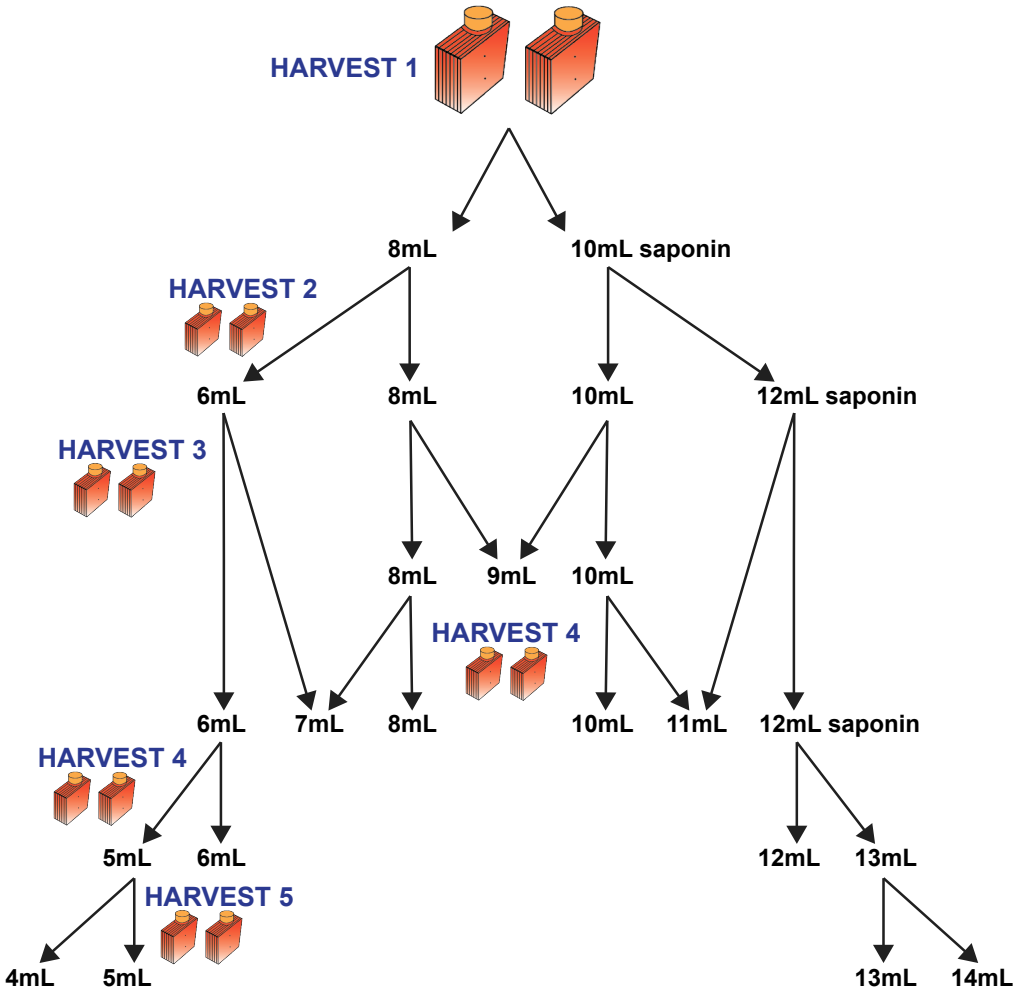

FIGURE 2.1 Flowchart of Saponin Batch Calibration

Saponin amounts for RBC lysis are empirically determined through pairwise comparison for each preparation/batch of saponin. 3 to 5 harvests and pairwise tests will be required to determine the ideal amount of saponin for a given batch. Volumes indicated on the flowchart are for the volume of 0.15% saponin (in Buffer A) to be added to parasites in Buffer A and to a total volume of 50mL. Harvest 1 should be tested with 8mL and 10mL saponin. Subsequent pairs for testing are determined by following the arrows on the flowchart: if 8mL yields the more active extract in Harvest 1, Harvest 2 will compare 8mL with 6mL saponin; if 6mL yields the more active extract in Harvest 2, Harvest 3 will compare 6mL with 7mL saponin, and so on, until a final value has been reached.

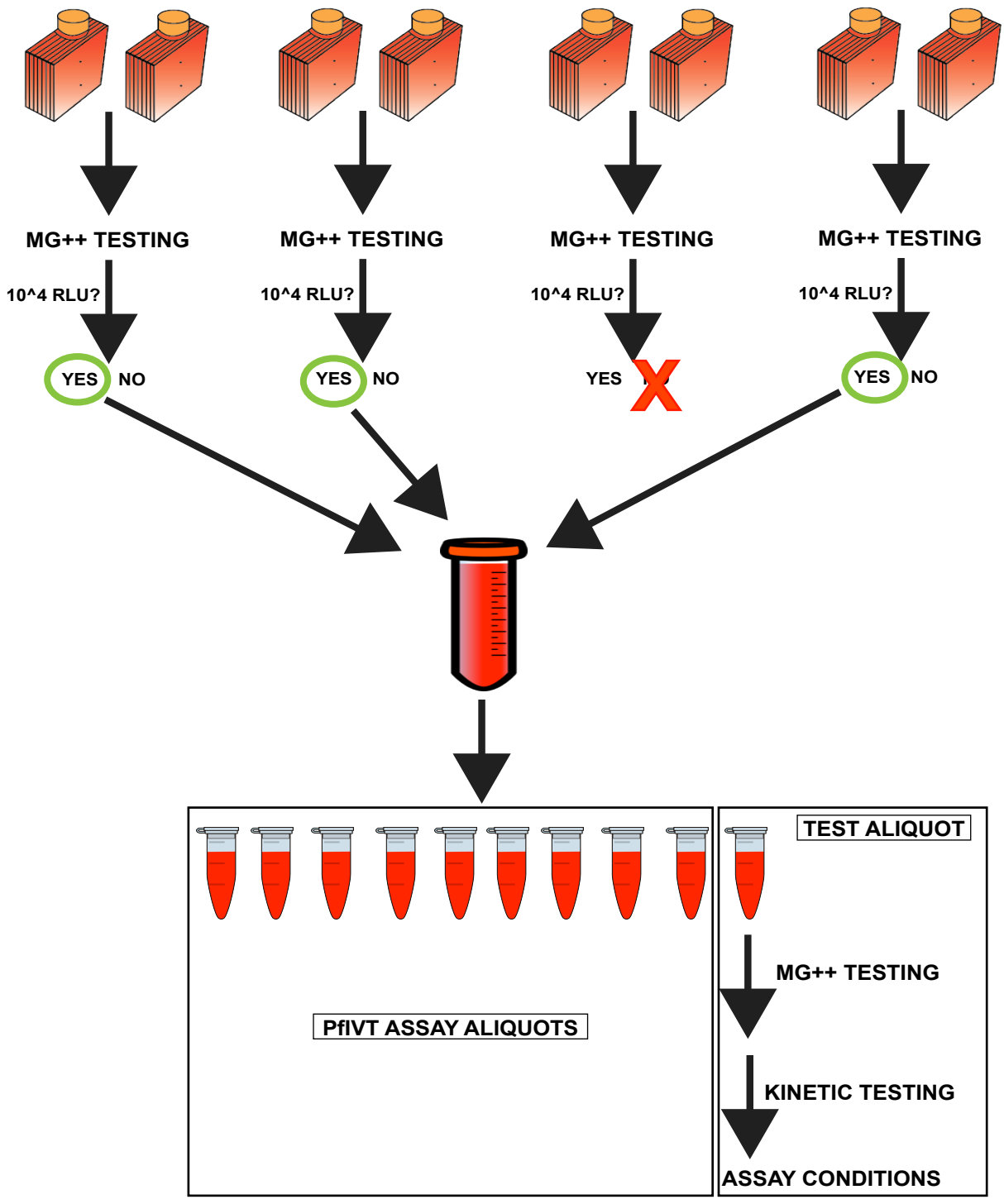

FIGURE 2.2 Flowchart for PfIVT extract quality control and pooling.

Individual harvests are first tested at different magnesium concentrations; those that achieve 10^4 RLU activity threshold are pooled. Pooled extract is aliquoted, and a test aliquot is utilized to first determine ideal magnesium concentration, then ideal incubation time. Remaining aliquots are utilized for PfIVT assays at the determined magnesium & kinetic conditions.

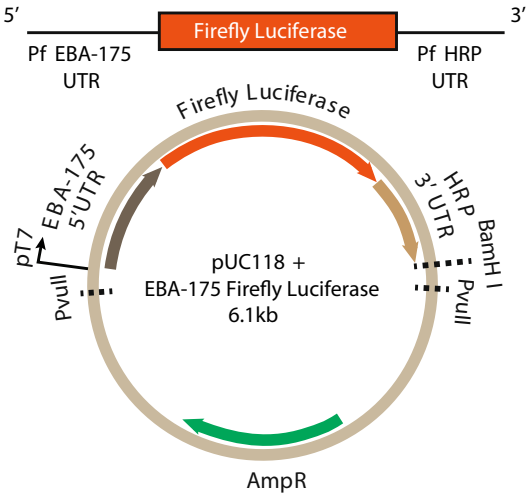

FIGURE 2.3 Control plasmid construct

Plasmid construct to generate mRNA transcript with Pf EBA-175 5'UTR, firefly luciferase open reading frame, and Pf HRP 3'UTR.

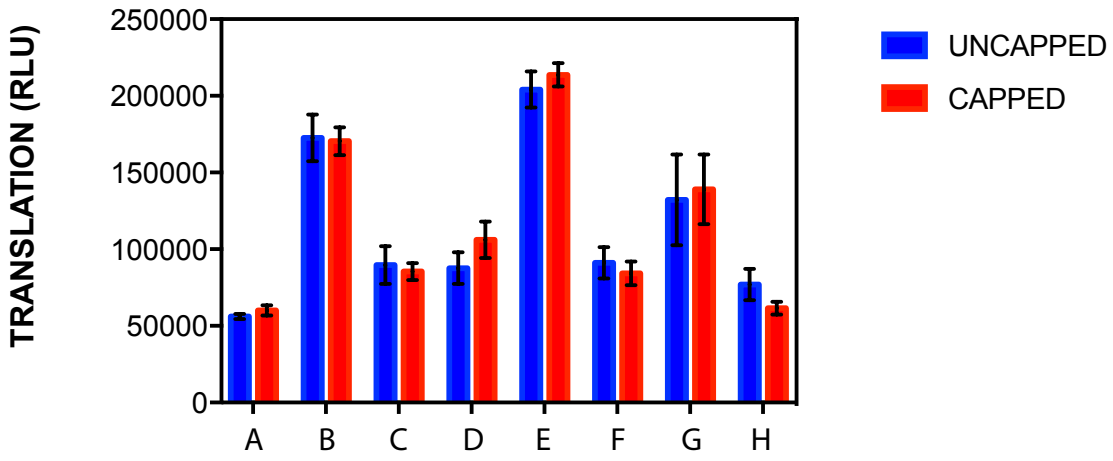

FIGURE 2.4 Comparison of capped an uncapped transcripts in the PfIVT assay.

Eight different firefly luciferase reporter constructs (A-H) were T7-transcribed without 5'Cap Each transcript was the divided into two pools: one remained uncapped (blue bars), and the other wascapped using the NEB Vaccinia Capping System (red bars). PfIVT assays were performed in duplicate, with all transcripts on the same plate, for direct comparison of uncapped and capped version of the same transcript.

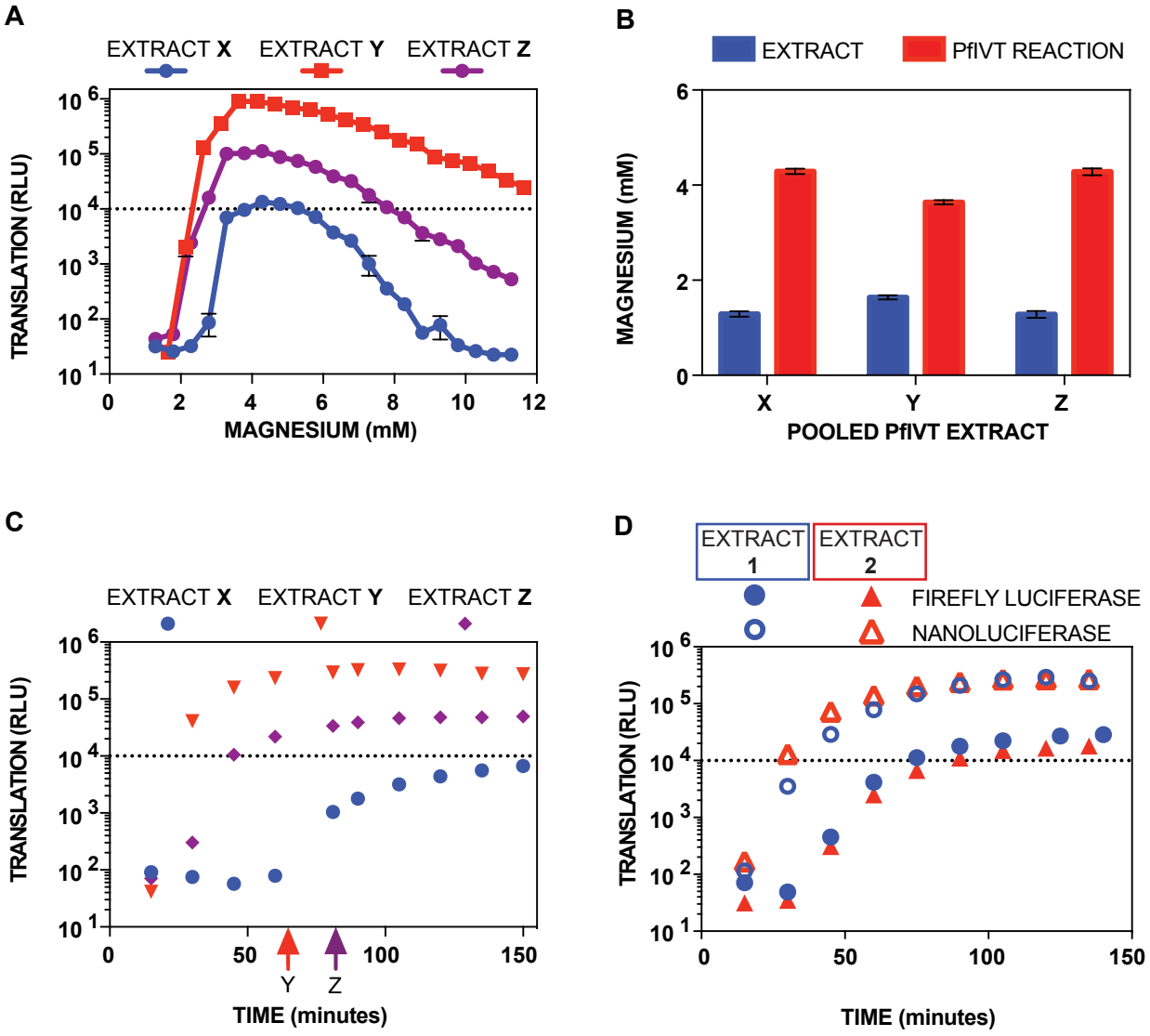

FIGURE 2.5 Optimization & quality control parameters of PfIVT extracts.

(A) Translational activity of 3 representative extracts (X, Y, and Z) over a range of reaction magnesium concentrations. **(B)** Measured basal extract magnesium concentration (blue bars) and optimum translation reaction magnesium concentration (red bars) for each of 3 representative PfIVT extracts (X, Y, and Z). **(C)** Kinetic curves for translational activity of each of 3 representative PfIVT extracts (X, Y, and Z) at the optimum reaction magnesium concentration shown in part B. **(D)** Kinetic curves for translational activity of each of 2 representative PfIVT extracts (1 and 2) with both firefly luciferase and nanoluciferase reporters. The dashed line at 10^4 relative luciferase units (RLU) represents the cutoff for acceptable translational activity for the assay. Extract X does not consistently meet the 10^4 RLU activity threshold and would not be used for PfIVT assays.

2.7 TABLES

TABLE 2.1 10X Translation Mix

Components to be gently combined on ice (DO NOT VORTEX!)

	STOCK []	10X TM []	100 µL
HEPES	1 M	20 mM	2 µL
KOAc	2 M	75 mM	3.75 µL
Mg(OAc)2	100 X	10 X	10 µL
DTT	0.1 M	2 mM	2 µL
ATP	0.1 M	5 mM	5 µL
GTP	0.1 M	1 mM	1 µL
Phosphocreatine	760 mM	200 mM	26.32 µL
Creatine Phosphokinase	5 mg/ml	2 µg/µL	40 µL
DEPC H_2O	-- --	-- --	9.93 µL

TABLE 2.2 PfIVT Master Mix for Initial Extract Quality Testing

Components to be gently combined on ice (DO NOT VORTEX!)
Adjust volume based on number of extracts to be tested.

	PfIVT Reaction	PfIVT Master Mix
	20μL TOTAL	x8.5 per extract
PfIVT Extract	14μL	--
10X TM	2μL	--
Amino Acids	1μL	8.5μL
FLUC mRNA (1μg/μL)	1μL	8.5μL
DEPC H_2O	2μL	17μL

TABLE 2.3 Magnesium Master Mix for Initial Extract Quality Testing

To be made for each concentration of magnesium.
Components to be gently combined on ice (DO NOT VORTEX!)
Adjust volume based on number of extracts to be tested.

	PfIVT Reaction	Magnesium Master Mix
	20μL TOTAL	x1.1 per extract
PfIVT Extract	14μL	--
10X TM	2μL	2.2μL
PfIVT Master Mix	4μL	4.4μL

TABLE 2.4 Pool Master Mix for Magnesium Testing of Pooled PfIVT Extracts

Components to be gently combined on ice (DO NOT VORTEX!)

	PfIVT Reaction	PfIVT Master Mix
	20μL TOTAL	x16
PfIVT Extract	14μL	224μL
10X TM	2μL	--
Amino Acids	1μL	16μL
FLUC mRNA (1μg/μL)	1μL	16μL
DEPC H_2O	2μL	--

TABLE 2.5 Magnesium Master Mix for Magnesium Testing of Pooled PfIVT Extracts

To be made for each concentration of magnesium.
Components to be gently combined on ice (DO NOT VORTEX!)

	PfIVT Reaction	Magnesium Master Mix
	20μL TOTAL	x3
Pool Master Mix	16μL	--
10X TM	2μL	6μL
DEPC H_2O	2μL	6μL

TABLE 2.6 Pool Master Mix for Kinetic Testing of Pooled PfIVT Extracts

Components to be gently combined on ice (DO NOT VORTEX!)

	PfIVT Reaction	PfIVT Reaction Master Mix
	20μL TOTAL	x16
PfIVT Extract	14μL	224μL
10X TM	2μL	32μL
Amino Acids	1μL	16μL
FLUC mRNA (1μg/μL)	1μL	16μL
DEPC H_2O	2μL	23μL

CHAPTER 3. CHARACTERIZATION OF THE PFIVT ASSAY AND DETERMINATION THAT NO CURRENT ANTIMALARIAL DRUGS DIRECTLY INHIBIT TRANSLATION

3.1 OVERVIEW, ABSTRACT, AND INTRODUCTION

3.1.1 Overview

In Chapter 2, I put forward a detailed view of the validation, optimization, and quality control of the *P. falciparum in vitro* translation (PfIVT) assay, as well as a practical guide and step-by-step protocols to enable use of the method by others. In this chapter, I present a thorough characterization of the PfIVT assay using a set of well-characterized tool compounds to demonstrate that:

(1) the PfIVT assay specifically measures activity of *P. falciparum* cytoplasmic ribosomes (as opposed to mitochondrial or apicoplast ribosomes),

(2) the PfIVT assay specifically identifies translational inhibitors that act directly upon the ribosome or associated translational apparatus, and correctly differentiates between these direct inhibitors of translation and inhibitors of other processes that often present as false-positives in historically-utilized indirect measures of translation such as S35-radiolabeled amino acid incorporation, and

(3) the PfIVT assay faithfully recapitulates the full process of translation, and properly identifies inhibitors of both the initiation and elongation stages of translation with a variety of mechanisms of action and binding sites.

Further, I utilize the assay to characterize currently approved clinical antimalarials with unknown, unclear, or disputed mechanisms of action, demonstrating

that none inhibit translation. This panel of antimalarials includes mefloquine, which was recently mischaracterized as a *P. falciparum* ribosomal inhibitor. In the addendum to this chapter, I put forth preliminary data suggesting an alternate mechanism of action for mefloquine.

3.1.2 Abstract

The continued specter of resistance to existing antimalarials necessitates the pursuit of novel targets and mechanisms of action for drug development. One class of promising targets consists of the 80S ribosome and its associated components comprising the parasite translational apparatus; thus, a greater understanding of protein synthesis and its regulation in the malaria parasite would advance the development of effective inhibitors. Research in this area has been limited by the lack of appropriate experimental methods, particularly a direct measure of parasite translation. We have recently developed and optimized the PfIVT assay, an *in vitro* method directly measuring translation in whole-cell extracts from the malaria parasite *Plasmodium falciparum*. Here, we present an extensive pharmacologic assessment of the PfIVT assay using a wide range of known inhibitors, demonstrating its utility for studying activity of both ribosomal and non-ribosomal elements directly involved in translation. We further demonstrate the superiority of this assay over a historically utilized indirect measure of translation, S35-radiolabel incorporation. Additionally, we utilize the PfIVT assay to investigate a panel of clinically approved antimalarial drugs, many with unknown or unclear mechanisms of action, and show that none inhibit translation,

reaffirming this mechanism of action to be a viable orthogonal drug target. Within this set, we unambiguously find that mefloquine lacks translation inhibition activity, despite having been recently mischaracterized as a ribosomal inhibitor. This work exploits a direct and reproducible assay for measuring *P. falciparum* translation, demonstrating its value in the continued study of protein synthesis in malaria and its inhibition as a drug target.

3.1.3 Introduction

Despite ongoing efforts in its treatment and prevention, malaria remains a severe global health burden, with nearly half the world's population at risk, and incidence of the disease actually increasing in the most recent years for which data are available (1). Though malaria-related mortality has continued to decrease, the rise in incidence is particularly concerning in light of reduced investment worldwide in combatting malaria, combined with climate change and geopolitical instability that may contribute to a resurgence of the disease (1). One compounding factor in the battle to eliminate malaria is the persistent emergence of drug resistance in the malaria parasite *Plasmodium falciparum* (1). As combination therapies are the main defense against resistance, an important focus in therapeutic development is the identification of compounds with unique targets and novel mechanisms of action that are unlikely to be precluded by existing resistance mutations. Medicines for Malaria Venture (MMV) has recently demonstrated the potential of efforts directed at novel targets; two drugs

currently showing great promise in clinical trials, SJ733 and cipargamin, inhibit the *P. falciparum* cation ATPase PfATP4, constituting a new class of drug (25,26).

One promising avenue for development of a novel target class is the inhibition of the *P. falciparum* ribosome, as well as other components of the translational machinery responsible for protein synthesis. Translation inhibitors have exhibited great clinical success as potent antibiotics, and in fact, several, including doxycycline and azithromycin, have found additional application as antimalarials, as they target ribosomes within the malaria parasite's mitochondria and apicoplast, leading to loss of function of these organelles (27,28). Interestingly, the *P. falciparum* cytoplasmic ribosome appears to occupy an evolutionary middle ground between prokaryotic and eukaryotic, differentiating it sufficiently from human ribosomes to yield a useful therapeutic window (28). Indeed, a potent and highly selective inhibitor of the *P. falciparum* ribosome, DDD107498, is currently in pre-clinical development, validating the potential of the *P. falciparum* translational apparatus as an effective target for antimalarial drugs and highlighting the need for further identification of such antimalarials (7).

To facilitate the identification of translation inhibitors, we previously developed a *P. falciparum* whole-cell extract-based *in vitro* translation assay (PfIVT), and successfully applied the technique to detect small molecule inhibitors in the MMV Malaria Box (8). More recently, it has been suggested that the widely used drug mefloquine may inhibit the 80S ribosome of *P. falciparum* (29). In addition, many currently approved antimalarial compounds lack a definitive mechanism of action, raising the possibility that some of these clinical therapies also act through inhibition of

translation. Here, we aimed to clarify which compounds truly exhibit inhibitory activity against the *Plasmodium falciparum* 80S ribosome and the associated translational apparatus. To do so, we compared a panel of antimalarial drugs (both clinical and pre-clinical) with well-characterized inhibitors of translation and other defined control compounds in the PfIVT assay, as well as in the S35-radioabel incorporation assay, a historically utilized indirect measure of translation. Importantly, we found that none of the current clinical therapeutics inhibited translation, including mefloquine. Regardless, testing of tool compounds shows that the PfIVT assay is capable of identifying not only translation inhibitors that directly interact with the ribosome, but also inhibitors of other non-ribosomal components of the translational machinery, demonstrating the broad utility of the assay for identifying novel malaria therapeutics that target *P. falciparum* translation.

3.2 RESULTS AND DISCUSSION

3.2.1 Results

3.2.1.1 Characterization of the PfIVT Assay and Comparison to Historically Utilized Measurement of Translation

Probing Different Stages of Translation in a P. falciparum Cellular Extract System Using Tool Compounds

The process of translation may be binned into three main phases: initiation, elongation, and termination (30,31). In eukaryotes, this process is carried out by the 80S ribosome, comprised of a small (40S) and large (60S) subunit (30,31). To further validate the PfIVT assay and investigate its capacity to interrogate the entirety of the normal activity of the 80S ribosome (and thus identify drugs inhibiting all steps of the process of translation), an extensive panel of previously characterized translational inhibitors was tested, both in the PfIVT assay, as well as in the historically utilized S35-radiolabelled amino acid incorporation assay. In contrast to the PfIVT assay, which directly measures activity of the 80S ribosome and the associated translational apparatus, S35 incorporation is an indirect measure of translation. Despite this, and the S35 incorporation assay's resulting sensitivity to changes to upstream and parallel pathways, which often generating ambiguous or misleading results, it has remained the standard assay for studying parasite translation in the absence of a better alternative (29).

The drugs tested in these assays included commercially available compounds that directly interact with the eukaryotic ribosome to inhibit translation initiation and/or elongation via a variety of mechanisms and binding sites, as well as several inhibitors of translation known to act upon non-ribosomal components of the translational machinery (TABLES 3.1 & 3.2). The eukaryote-specific inhibitors bruceantin and verrucarin A inhibit translation initiation through binding of mutually exclusive sites (31–34). Suramin, also a specific inhibitor of the eukaryotic ribosome, inhibits both initiation and elongation through binding of multiple sites on the 40s, 60S and 80s ribosomes (35). The eukaryote-specific elongation inhibitors tested are also distinct in their activities:

cycloheximide and lactimidomycin overlap in their binding of the ribosome A-site, but differences in size and side-chains yield unique effects; anisomycin also overlaps cycloheximide's binding site, but the two drugs bind the ribosome in distinct rotational conformations at different steps of elongation; homoharringtonine binds the A-site, but specifically inhibits re-initiating ribosomes; and nagilactone C inhibits both eEF-1α-dependent aminoacyl-tRNA loading and peptidyl transferase activity (31,32,36–38). Halofuginone, also a specific inhibitor of eukaryote translation, does not interact with the ribosome, but instead inhibits glutamyl-prolyl-tRNA synthetase (39). Puromycin was the sole pan-inhibitor tested, and acts as a tRNA mimetic that is incorporated into the nascent polypeptide chain, leading to its premature termination (40,41). Negative controls were thiostrepton, a specific inhibitor of prokaryotic translation initiation and elongation; actinomycin D, an inhibitor of RNA polymerase II; tubercidin, an adenosine mimetic; and thapsigargin, a sarco/endoplasmic reticulum ATPase (SERCA) inhibitor (42–51).

After determining the EC50 of each drug for the *P. falciparum* W2 strain in a 72-hour parasite growth assay, the drugs were characterized in both the S35 incorporation and PfIVT assays (TABLE 3.3, FIGURES 3.1 & 3.2). Drugs were tested in the S35 and PfIVT assays at 0.1-, 1-, 10-, and 100-fold their determined growth assay EC50 in W2 parasites, except in cases where the highest concentration was constrained by solubility or available stock solution. The translation initiation inhibitors bruceantin and verrucarin A were both potent (nanomolar) inhibitors of S35 incorporation and PfIVT (FIGURE 3.1). All translation elongation inhibitors (anisomycin, cycloheximide, homoharringtonine, lactimidomycin, and nagilactone C) also strongly inhibited both S35 incorporation and

PfIVT (FIGURE 3.1). Cycloheximide was additionally tested at 1000-fold its EC50, as it did not inhibit S35 incorporation at the lower concentrations tested, but did at this higher concentration (FIGURE 3.3). Suramin, which has been shown to inhibit both translation initiation and elongation, robustly inhibited PfIVT, but not S35 incorporation, likely due to poor cell permeability and the short timeframe of the S35 assay (2-hour drug pre-incubation followed by 2-hour radiolabel incorporation) (FIGURE 3.1). The tRNA mimetic puromycin, which induces premature termination of nascent polypeptides, inhibited both S35 incorporation and PfIVT with similar efficacy (FIGURE 3.1). Elucidating an even greater range of utility for the PfIVT assay, we found it to be capable of identifying inhibitors of non-ribosomal components of translation. The glutamyl-prolyl-tRNA synthetase inhibitor halofuginone inhibits both S35 incorporation and the PfIVT assay (FIGURE 3.1). In sum, these data demonstrate the ability of the PfIVT assay to interrogate both direct ribosomal activity, as well as extra-ribosomal components of the translational machinery.

The P. falciparum In Vitro Translation Assay Measures Activity of Cytoplasmic Ribosomes

Importantly, all of the eukaryotic ribosome-specific inhibitors, which therefore should inhibit only *P. falciparum* cytoplasmic and not apicoplast or mitochondrial ribosomes, inhibited the PfIVT assay, with several achieving complete or near complete inhibition of translation (suramin, anisomycin, lactimidomycin, nagilactone C) (FIGURE 3.1). We also tested several prokaryotic ribosome-specific translation inhibitors,

including thiostrepton, doxycycline, and azithromycin, (FIGURES 3.2 & 3.4, TABLES 3.1 & 3.3) (52,53). While each inhibited S35 incorporation to varying degrees at concentrations above their respective EC50s, thiostrepton achieved negligible inhibition of translation in the PfIVT assay at any concentration tested, (FIGURE 3.2). Doxycycline and azithromycin, both of which are known to have inhibitory effects on apicoplast and/or mitochondrial ribosomes, strongly inhibited the PfIVT assay, but only at exceptionally high (millimolar) concentrations (FIGURE 3.4A) (27,28,54). Note that it is well established that both drugs exhibit a delayed inhibitory effect in growth assays, and thus EC50 values are usually determined in 96-hour assays comprising 2 parasite life cycles, rather than the single-cycle assay utilized in this study (27,28,54). At these very high concentrations, the inhibitory effect may not be physiologically relevant, or may be due to non-specific inhibition of the cytoplasmic ribosomes. Together, these results demonstrate that the vast majority of translation measured in our PfIVT system is the product of cytoplasmic ribosomal activity.

The S35 Incorporation Assay Is Not a Reliable Indicator of Direct Translation Inhibition

Although it is well documented in other model systems (i.e. yeast) that the S35-radiolabeled amino acid incorporation assay is an indirect measure of translation and can, as such, generate many misleading artifacts, this has not yet been characterized carefully with respect to *Plasmodium spp.* (55,56). Despite this, several studies in *Plasmodium* have relied on this indirect measure as a primary readout of translation

(29,57). To address this and further determine the specificity of the PfIVT assay relative to the S35 uptake assay, we tested a panel of small molecules that are known to inhibit cellular processes other than translation (TABLES 3.2 & 3.3). Not surprisingly, actinomycin D, an inhibitor of transcription targeting RNA Polymerase II, and the SERCA inhibitor thapsigargin both exhibited strong inhibition in the S35 incorporation assay, but had no effect in the PfIVT assay (FIGURE 3.2). Tubercidin, an adenosine mimetic, had a modest inhibitory effect on S35 incorporation, but, again, negligible effect in the PfIVT assay (FIGURE 3.2). These data confirm that the PfIVT assay directly measures translation, and highlight the lack of translation specificity of the S35 incorporation assay.

3.2.1.2 Screen of Existing Antimalarial Drugs to Identify P. falciparum Translation Inhibitors

Analysis of Clinically Approved Antimalarials Reveals That None, Including Mefloquine, Inhibit the 80S Ribosome

We next sought to test a panel of clinically approved antimalarial drugs with undefined or disputed mechanisms of action, to determine whether any might act through direct inhibition of translation, subjecting these drugs to the same battery of assays described above (*P. falciparum* growth, PfIVT, and S-35 incorporation)(TABLES 3.3 & 3.4). Chloroquine and piperaquine were mild inhibitors of the S35 incorporation assay at the highest drug concentrations tested (FIGURE 3.5). Quinine, lumefantrine,

primaquine, monodesethyl amodiaquine (the active metabolite of amodiaquine), and dihydroartemisinin were moderate-to-strong inhibitors of the S35 incorporation assay (FIGURE 3.5). SJ733, an inhibitor of the sodium transporter PfATP4, and a clinical candidate currently in Phase I trials, exhibited strong inhibition in the S35 incorporation assay (FIGURE 3.5). Notably, none of these antimalarial drugs inhibited the PfIVT assay. However, primaquine may warrant follow-up testing in the PfIVT assay if its active metabolite can be isolated, given its moderate activity in the S35 incorporation assay (58).

We also included several drugs (clinical and pre-clinical) that have recently been reported to inhibit translation (TABLES 3.3 & 3.4)(7,8,29). The MMV008270 was a moderate inhibitor of the S35 incorporation assay, while mefloquine and DDD107498 robustly inhibited S35 incorporation (FIGURE 3.5). Strikingly, while DDD107498 and MMV008270 inhibited the PfIVT assay, mefloquine failed to do so (FIGURE 3.5). Interestingly, MMV008270 was an exceptionally effective inhibitor of translation in the PfIVT assay at all concentrations tested, significantly outperforming the S35 incorporation assay (FIGURE 3.5). These data reveal that mefloquine has recently been mischaracterized as a ribosome inhibitor through use of the S35 incorporation assay, when it does not, in fact, directly inhibit translation (29).

To further validate the PfIVT data regarding mefloquine, we repeated the PfIVT assay, alongside a commercially available rabbit reticulocyte *in vitro* translation assay (RRIVT), with a full titration of drug to determine half maximal effective values for both mefloquine and DDD107498 (FIGURE 3.6). As expected, the positive control cycloheximide was a robust inhibitor of both translation systems (PfIVT IC50: 31.91nM,

RRIVT IC50: 37.8nM), while DDD107498 was a potent inhibitor of *P. falciparum*, but not rabbit reticulocyte translation, confirming the reported high *P. falciparum* selectivity of DDD107498 (PfIVT IC50: 60.5nM)(FIGURE 3.6). In contrast, mefloquine failed to inhibit in either the PfIVT or RRIVT assay, even at concentrations as high as 20uM (FIGURE 3.6). The reported binding site of mefloquine to the 80S ribosome is on the highly conserved ribosomal protein uL13; if this were indeed the active binding site of the drug, mefloquine should inhibit the RRIVT assay (59). To rule out the possibility that mefloquine solubility may be a confounding factor in the IVT assays, completed PfIVT reactions with a dilution series of mefloquine or DMSO control were centrifuged at high speed, sterile-filtered, and the resulting supernatant was used as the input for an *in vivo* growth assay. EC50 values were comparable between the IVT reaction supernatant containing mefloquine (12.31nM) and mefloquine alone (4.17nM), thus demonstrating that mefloquine is soluble in the PfIVT assay (FIGURE 3.7). These data make clear that mefloquine does not act through inhibition of the *P. falciparum* ribosome, nor through other direct inhibition of the translational machinery.

3.2.2 Discussion

This work presents an extensive dissection and validation of the whole-cell extract-derived PfIVT assay, the only reported direct measure of *P. falciparum* translation to date. Through probing the assay with numerous small molecule inhibitors of translation, exhibiting a diversity of binding sites and mechanisms of action, as well as a variety of well-characterized tool compounds inhibiting non-translational pathways,

we demonstrate that the PfIVT assay specifically measures *P. falciparum* cytoplasmic ribosome activity. *In vitro* translation extracts are inherently difficult to make, and even more so for an intraerythrocytic parasite. However, when subjected to stringent quality control and careful optimization, the PfIVT assay reliably and specifically identifies inhibitors of translation initiation and elongation, as well as inhibitors of non-ribosomal proteins necessary for translation, such as tRNA synthetase.

The PfIVT assay is particularly valuable to the study of *P. falciparum* translation as a direct measure of translation, as opposed to the indirect measures to which the field has historically been constrained, such as incorporation of radiolabeled amino acids *in vivo*. Importantly, our data show the PfIVT assay to be more significantly more accurate, and in some cases more sensitive, than S35-radiolabel incorporation in identifying small molecule inhibitors of translation. Indeed, the PfIVT assay specifically identified all eukaryotic translation inhibitors tested, while S35-radiolabel incorporation was prone to false-positives. We found that none of the clinically approved antimalarials tested were inhibitors of translation, emphasizing the potential for translation as a useful therapeutic target, as there is unlikely to be pre-existing mechanism-specific resistance to any identified candidates resulting from use of these drugs. It is notable that mefloquine, in contrast to other previously reported translation inhibitors, did not exhibit any inhibitory activity. Mefloquine was likely mischaracterized as an 80S ribosome inhibitor through a combination of non-specific inhibition of S35 incorporation, as well as artifacts arising from cryo-EM structures obtained under the non-physiologic condition of 10mM magnesium – well above the ~4mM magnesium that we have found to be optimal for translation (FIGURE 2.5A&B)(29).

While the PfIVT assay exhibits clear benefits over existing methodologies for the study of *P. falciparum* translation, we acknowledge that the technique has several limitations. As is the case with *in vitro* translation systems in other organisms, the current assay is likely biased toward the study of non-cap-dependent initiation and elongation. We utilized uncapped mRNA in this study to focus specifically on activity of the 80S ribosome itself, rather than the cap-recognition apparatus. It is possible that utilization of capped mRNA in future studies would facilitate interrogation of cap-dependent translation initiation. Likewise, there are few characterized pharmacologic inhibitors of eukaryotic translation termination, none of which are currently commercially available; thus, the PfIVT system, as described, may not be sensitive to all specific inhibitors of translation termination. Additionally, some translation inhibitors, such as homoharringtonine, demonstrated greater potency in the S35 incorporation and growth inhibition assays than in PfIVT. Such variation between the two assays may suggest off-target effects of these drugs, or differences between whole living cells and cellular extracts.

Determining the true molecular targets of antimalarials is critical to improved therapeutic development. Exploiting differences between *P. falciparum* and mammalian ribosomes remains a promising avenue, as evidenced by the potent and discriminating drug DDD107498. Here, we have shown that orthogonal biochemical assays may be used to test hypotheses generated by structural data and cell-based assessments. Our investigation of mefloquine reaffirms that direct functional measurements of drug activity are critical to identifying the genuine molecular targets of drugs. Importantly, we show that the PfIVT assay is a uniquely direct measure of *P. falciparum* translation that can

be used to elucidate such drug studies and facilitate a better understanding of the specifics of *P. falciparum* protein synthesis, with potentially great consequences for human health.

3.3 METHODS AND MATERIALS

3.3.1 Drug Stocks

In vivo growth and *in vitro* translation measurements were performed using the same drug dilutions. The antimalarial drugs chloroquine, dihydroartemisinin, lumefantrine, monodesethyl amodiaquine, piperaquine, primaquine, and quinine were a generous gift from Dr. Phil Rosenthal of UCSF. SJ733 was generously provided by Dr. Kip Guy of St. Jude Children's Research Hospital. All other compounds were purchased from the indicated vendors: DDD107498 (Apexbio #A8711-5), mefloquine hydrochloride (Sigma-Aldrich #M2319), doxycycline hyclate (Sigma-Aldrich D9891), emetine (Sigma-Aldrich #E2375), cycloheximide (Fisher #AC35742-0010), MMV008270 (Vitas-M Laboratory #STK591252), actinomycin D (Sigma-Aldrich #A1410), tubercidin (Sigma-Aldrich #T0642), thapsigargin (Sigma-Aldrich #SML1845), ionomycin (Sigma-Aldrich #407951), thiostrepton (Sigma-Aldrich #598226), bruceantin (Toronto Research Chemicals #B689310), verrucarin A (Sigma-Aldrich #V4877), anisomycin (Sigma-Aldrich #A5862), homoharringtonine (Sigma-Aldrich #SML1091), lactimidomycin (EMD Millipore #506291), nagilactone C (BOC Sciences #24338-53-2), suramin sodium salt

(Sigma-Aldrich #S2671), puromycin (Thermo Fisher #A1113803), halofuginone (Sigma-Aldrich #32481), azithromycin (Sigma-Aldrich #75199).

3.3.2 Plasmodium falciparum Strain and Culturing

Plasmodium falciparum W2 (MRA-157) was obtained from MR4. Parasites were grown in human erythrocytes (2% hematocrit) in RPMIc (RPMI 1640 media supplemented with 0.25% Albumax II (GIBCO Life Technologies), 2g/L sodium bicarbonate, 0.1mM hypoxanthine, 25mM HEPES (pH 7.4), and 50µg/L gentamicin), at 37°C, 5% O_2, and 5% CO_2. Cells were synchronized with 5% sorbitol treatment for two generations to achieve high synchronicity.

3.3.3 Growth Inhibition Assays

2µL of serial drug dilutions in 100% DMSO were dispensed in triplicate to 96-well plates utilizing the LabCyte ECHO acoustic liquid handler. 198µL of *P. falciparum* W2 cultures were added. Growth was initiated with ring-stage parasites at 0.8% parasitemia and 0.5% hematocrit. Plates were incubated at 37°C, 5% O_2, and 5% CO_2 for 72 hours. Growth was terminated by fixation with 1% formaldehyde, and parasitized cells were stained with 50nM YOYO-1 (Invitrogen). Parasitemia was determined by flow cytometry on the BD LSRII, analyzed using FlowJo software version 10, and EC50 were curves plotted by GraphPad Prism. Two biological replicates were performed in triplicate for each drug.

3.3.4 Generation and Quality Control of Extracts for Plasmodium falciparum In Vitro Translation Assay

For PfIVT harvests, one liter of synchronized parasite culture in 2-4% hematocrit was grown in two 500mL HYPERFlask M vessels (Corning), and media was changed every 8-12 hours, with the final media change at 4-8 hours prior to harvest. Parasites were harvested in the late trophozoite stage at 15-20% parasitemia by centrifugation for 5 minutes at 1500×*g* at room temperature, followed by removal of media and addition of ice-cold 0.025-0.05% final saponin in Buffer A (20mM HEPES pH 8.0, 2mM $Mg(OAc)_2$, 120mM KOAc). Due to variations between and within lots, saponin stocks were prepared in large volumes, aliquoted, and stored at -20°C. Percentage utilized for each batch of aliquots was determined empirically through pairwise testing of concentrations (1 for each HYPERFlask) and assessed via resulting activity of PfIVT extracts. Saponin-lysed pellets were centrifuged at 4°C and 10,000×*g* for 10 minutes and washed twice with ice-cold Buffer A. Supernatant was carefully removed, and washed pellets were resuspended in an equal volume of Buffer B2 (20mM HEPES pH8.0, 100mM KOAc, 0.75mM $Mg(OAC)_2$, 2mM DTT, 20% glycerol, 1X EDTA-free protease inhibitor cocktail (Roche)), flash-frozen, and stored in -80°C freezer until the sample was ready to homogenize.

Frozen pellets were thawed on ice and added to a 3-mL Luer lock syringe, which was then secured onto a pre-chilled cell homogenizer containing a 4μm-clearance ball bearing (Isobiotec, Germany) that was pre-washed with ice-cold Buffer B2.

Homogenate was passed between two syringes 20 times on ice, either by hand or by use of a custom robot built to accommodate the cell homogenizer (23). Lysate was immediately centrifuged at 4°C and 16,000×*g* for 10 minutes, and the supernatant (the resulting PfIVT extract) was transferred to a fresh tube, with a small (100μL) aliquot set aside for activity testing. Extracts and test aliquots were flash-frozen and stored at -80°C. Test aliquots from multiple harvests were thawed on ice and tested in batches in the PfIVT assay (see below) across a small range of magnesium concentrations with a 2-hour incubation time, using a firefly luciferase reporter. Extracts that surpass the activity threshold of 10^4 relative luciferase units (RLU) were then thawed on ice and combined to generate large volume pools. Extract pools were flash-frozen in 200μL aliquots and stored at -80°C. Extract pools were tested across a range of magnesium concentrations via PfIVT assay to determine the optimum magnesium concentration. Once magnesium concentration has been determined, pools are then tested in the PfIVT assay in 15-minute incubation time points up to 150 minutes to determine the kinetics of the extract pool, and thus the appropriate incubation time for the pool (~75-80% of maximum signal, within the linear range of the extract's kinetic curve). Kinetics must be separately assessed for each reporter used (i.e. if a nanoluciferase reporter is used instead of firefly luciferase).

3.3.5 Plasmodium falciparum In Vitro Translation Assay

P. falciparum in vitro translation (PfIVT) reactions were carried out in skirted V-bottom 96-well PCR plates (BioRad) and sealed with adhesive aluminum foil plate seals

(Beckman Coulter, Indianapolis, IN, USA). 200nL of drug in 100% DMSO was dispensed in duplicate to appropriate wells of the plate utilizing a Labcyte ECHO acoustic liquid handler. 19.8μL of PfIVT reaction mix (per 20μL: 14μL extract, 1μg T7-transcribed firefly luciferase mRNA, 10μM amino acid mixture, 20mM HEPES/KOH pH 8.0, 75mM KOAc, 2mM DTT, 0.5mM ATP, 0.1mM GTP, 20mM creatine phosphate, 0.2μg/μl creatine kinase, and the appropriate amount of $Mg(OAc)_2$ as determined for the particular pool of extract) was then dispensed to each well using Rainin E4 12-channel electronic pipettes (Rainin Instruments, Oakland, CA, USA). Reactions were incubated at 37°C for the appropriate amount of time as determined for the particular pool of extract. After incubation, the reactions were placed on ice, then quenched through transfer to a 96-well LUMITRAC 200 flat-bottom white assay plate (Greiner Bio-One, Monroe, NC, USA) containing 2μL of 50μM cycloheximide (dispensed using the Labcyte ECHO), then immediately centrifuged to combine the PfIVT reaction with the cycloheximide for a final concentration of 5μM cycloheximide. Reactions were assayed using the Promega GloMax-Multi + microplate reader with a 3-second delay and 3-second integration after addition of 200μL firefly luciferin reagent dispensed at a speed of 200μL/second (firefly luciferin reagent: 20mM Tricine, 2.67mM $MgSO_4 \times 7H_2O$, 0.1mM EDTA, 33.3mM DTT, 530μM ATP, 270μM Acetyl CoEnzyme A, 1mM D-Luciferin, 265μM Magnesium Carbonate Hydroxide pH 8.15). Three biological replicates were performed in duplicate for each drug. IC50 curves were plotted by GraphPad Prism.

3.3.6 Rabbit Reticulocyte In Vitro Translation Assay

Rabbit reticulocyte (Retic Lysate IVT Kit, Thermo Fisher Scientific, Waltham, MA, USA) in vitro translation assays were carried out with the following components in 20µL: 0.5µL 20× translation mix minus methionine, 0.5µL 20× translation mix minus leucine, 1 µg T7 transcribed firefly luciferase mRNA, 7µL reticulocyte lysate, and DEPC H_2O to final volume for 5–20 minutes at 37 °C. After incubation, the reactions were quenched with 5µM cycloheximide and assayed in the same manner as the *Plasmodium* lysates. IC50 curves were plotted by GraphPad Prism.

3.3.7 S35 Incorporation Assays

Parasite Purification:

Synchronized parasites were cultured in 2% hematocrit at 10-15% parasitemia, and MACS purified at the late trophozoite stage to remove uninfected erythrocytes using standard protocols. In brief, at least two LD MACS Separation columns (Miltenyi Biotech) per 50mL of culture were washed with 1.25mL of pre-warmed RPMIc. Next, cultures were added to the columns 5mL at a time and allowed to gravity filter at 37oC. Finally, the columns were rinsed with 2.5mL of pre-warmed RPMIc, removed from the magnetic stand, and eluted with 2mL of pre-warmed RPMIc.

Drug Treatment and S35 Labeling:

1μL of drug in 100% DMSO was dispensed to each well of a 96-well round-bottom culture plate utilizing the Labcyte ECHO acoustic liquid handler. $2x10^7$ MACS-purified parasites in 199μL of RPMIc were then added to each well. Parasites were incubated with drug for 2 hours at 37°C, 5% CO_2, 5% O_2. Next, samples were transferred from 96-well plates to 1.5mL screw-cap microfuge tubes. 35μCi of EasyTag™ Express S35 Protein Labeling Mix (Perkin Elmer) diluted to 10μL with RPMIc was added to each tube. Reactions were incubated at 37°C with mild shaking for 2 hours.

Washing and Lysis:

After incubation, cells were pelleted and 160μL of supernatant was removed. Parasites were then washed with 200μL of ice-cold PBS containing 50μM cycloheximide four times. After the final wash, all supernatant was removed and samples were resuspended in 15μL of 2X SDS buffer (100mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 0.1M DTT). Samples were boiled at 98°C for 5 minutes and stored at -20°C.

Scintillation Counting:

Samples were thawed at room temperature, boiled for 5 minutes at 98°C, and spun at max speed in a tabletop microcentrifuge for 10 minutes. 10μL of supernatant per sample was pipetted onto a 0.45μm nitrocellulose membrane (HAWP02400 from

Millipore) and allowed to dry completely. Each membrane was washed 4 times with 15mL of TBS-T then placed in a 20mL HDPE scintillation vial (Fisher Scientific) with 8mL of Ecoscint A scintillation fluid (National Diagnostics). S35 counts were measured for 1 minute using a Beckmann Coulter LS 6500 Multi-purpose Scintillation Counter. Three biological replicates were performed for each drug condition.

3.3.8 Mefloquine Solubility Assay

PfIVT extracts were incubated with a dilution series of mefloquine or DMSO control for 90 minutes. All PfIVT conditions were the same as above, except without addition of cycloheximide to stop translation. Reactions were centrifuged at 16,100x*g* for 10 minutes at room temperature; resulting supernatant was then filtered and added to cultures for the *P. falciparum* growth inhibition assays as described above.

3.4 ADDENDUM: MEFLOQUINE AND CALCIUM HOMEOSTASIS

3.4.1 Introduction and Rationale

In light of the fact that mefloquine does not directly inhibit the *P. falciparum* 80S ribosome, nor any other component of the translational apparatus, we sought to determine its true target. Transcriptional profiling of neuronal response to mefloquine demonstrated enrichment for regulatory sequences of the unfolded protein response (UPR), induction of endoplasmic reticulum (ER) stress proteins, and down-regulation of

ion channels; all pointing to disruption of calcium homeostasis (60,61). Several studies of mefloquine in other organisms, as well as *P. falciparum,* have implicated production of reactive oxygen species (ROS), which may be the result of ER stress and modulation of calcium, in mefloquine-induced toxicity (60–66). Still other studies have shown that mefloquine inhibits activity of non-receptor tyrosine kinases whose activity is also strongly affected by cytosolic calcium (67–70).

Inhibitors of sarco/endoplasmic reticulum ATPase (SERCA), such as thapsigargin, induce efflux calcium from sarco/endoplasmic reticulum stores, disrupting intracellular calcium homeostasis (51). Moreover, SERCA inhibitors are a classic example of compounds that yield false-positive results in the S35 radiolabel incorporation assays; disruption of the sarco/endoplasmic reticula induces a stress response, which in turn results in a downstream inhibition of protein synthesis. In fact, we saw in our own work that thapsigargin inhibits the S35 assay but has no effect on PfIVT, similar to mefloquine (FIGURES 3.2 & 3.5). In addition, mefloquine has been noted in the literature to affect multiple ATPases (71,72). Many ATPase and GTPase proteins are closely structurally related. While the study claiming direct inhibition of the *P. falciparum* 80S ribosome by mefloquine was incorrect in its conclusion, the related data suggesting an interaction between mefloquine and a ribosomal GTPase may actually highlight an off-target effect of mefloquine related to its true activity (29).

Calcium homeostasis is of crucial importance for *P. falciparum* biological activity and progression through blood stage, and any perturbation to this homeostasis could be very detrimental to the parasite (thereby yielding a potential therapeutic window) (73). Given the data suggesting a role for mefloquine in calcium homeostasis, thereby

indirectly inhibiting translation, we sought to determine whether mefloquine directly alters calcium homeostasis within *P. falciparum* parasites.

3.4.2 Results and Discussion of Findings

Mefloquine Disrupts Calcium Homeostasis at High Concentration

To determine whether mefloquine disrupts calcium homeostasis in intraerythrocytic *P. falciparum* parasites, we utilized a ratiometric fluorescent calcium indicator, Fura-2 AM. Parasites were pre-loaded with indicator, prior to drug treatment, in the presence of probenecid to prevent extrusion of indicator from parasite cytoplasm. Treatment with the calcium ionophore ionomycin induced a rapid and sustained increase of cytoplasmic calcium (FIGURE 3.8A). In contrast, treatment with negative control SJ733, a PfATP4 inhibitor, had no effect on calcium levels (FIGURE 3.8A). Treatment with the SERCA inhibitors thapsigargin and cyclopiazonic acid (CPA) increased cytoplasmic calcium, but to a lesser degree, and with a different kinetic profile, than ionomycin (FIGURE 3.8A & B). Mefloquine treatment induced a large increase in cytoplasmic calcium, but only at the highest drug concentration tested (FIGURE 3.8A). Interestingly, while the magnitude of the mefloquine-induced increase in cytoplasmic calcium was greater than that seen with ionomycin, the kinetics of the increase were most similar to those observed at the highest concentration of thapsigargin.

It is important to note that the effect of mefloquine on *P. falciparum* cytoplasmic calcium levels is observed only at very high concentration. However, in this particular experiment, very high concentrations of the various controls were also required to yield a significant effect. Given the extremely rapid effect of mefloquine, it is likely that any mefloquine-induced changes in calcium are through direct interaction with its target, rather than indirect. It is also worth noting that neuronal cells are particularly sensitive to calcium homeostasis, as calcium acts as a neurotransmitter, and thus alterations in calcium levels as a result of mefloquine treatment could account for the neurotoxic side effects of the drug seen in humans (74,75). This data is very preliminary, and requires further optimization, as well as observation over different time courses, however, it provides an interesting insight into a potential mechanism of action for mefloquine.

3.4.3 Materials and Methods

Reagents

Calcium indicators and drugs were purchased from the following vendors: Fura-2 AM (Thermo Fisher #F1221), ionomycin (Sigma-Aldrich #407951), cyclopiazonic acid (Sigma-Aldrich #C1530), probenecid (Thermo Fisher #P36400), Pluronic F-127 (Thermo Fisher #P3000MP).

Fura-2 AM Calcium Assays

Synchronous cultures of *P. falciparum* W2 strain blood stage parasites were MACS purified at the late trophozoite stage to isolate trophozoites. Intraerythrocytic parasites were washed 3 times with pre-warmed HBSS Loading Buffer (25mM HEPES, 121mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 10mM glucose, 0.25% BSA, 5mM NaHCO3, pH7.4). Parasites were resuspended to a concentration of 1E8 cells/ml in HBSS Loading Buffer + 2mM probenecid. Equal volumes of Pluronic F-127 and Fura-2 AM stock (2.5mM stock in DMSO) were pre-mixed, then this mixture was added to the resuspended parasites to a final concentration of 5uM Fura-2 AM (making sure to set aside an aliquot for a no Fura-2 AM control!). Parasites were then incubated at 37°C, 5% O2, and 5% CO2 for 50 minutes in a 6-well plate (2mL per well), with gentle mixing every 15 minutes. Parasites were then washed 3 times with bicarbonate-free RPMIc (RPMI 1640 media supplemented with 0.25% Albumax II (GIBCO Life Technologies), 0.1mM hypoxanthine, 25mM HEPES (pH 7.4), 50µg/L gentamicin, pH7.4) + 2mM probenecid. Parasites were allowed a 20-minute recovery at 37°C, 5% O2, and 5% CO2. Parasites were then briefly centrifuged, RPMI was removed, and parasites were resuspended in HBSS Loading Buffer + 2mM probenecid.

100µL labeled parasites were then distributed to wells of a clear-bottom black 96-well assay plate for fluorescent readings. Baseline fluorescence readings at 340ex/510em and 380ex/510em were taken (at 37°C) for each sample prior to drug addition. 100µL 2X drug stock or DMSO control was quickly added and gently pipetted to mix. Fluorescence readings at 340ex/510em and 380ex/510em were taken (at 37°C)

every 7 seconds for a total of 11.5 minutes (~70 seconds prior to addition of drug, then ~10.3 minutes following drug addition). 340/380 ratios were then calculated (to account for differences in loading and photobleaching), and ratios were then normalized to DMSO controls, and plotted using GraphPad Prism.

3.5 FIGURES

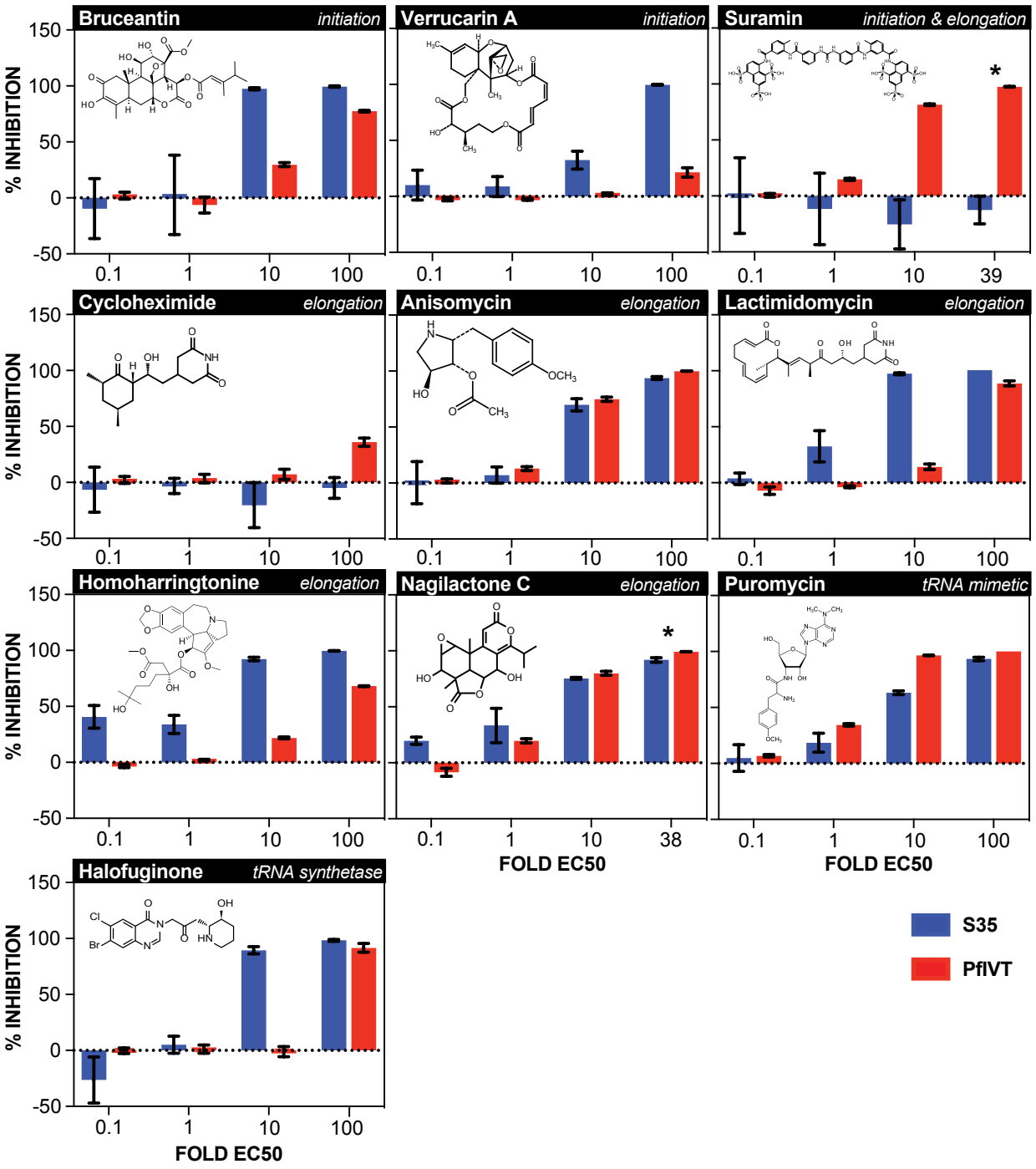

FIGURE 3.1 Dose-dependent inhibition of S35 incorporation and PfIVT by eukaryotic translation inhibitors.
Dose-dependent inhibition, calculated as % inhibition, of S35 incorporation (blue bars) and PfIVT assays (red bars) by eukaryotic translation inhibitors. Name of compound, mechanism of action, and molecular structure are displayed at top of each graph. Compounds were tested at 0.1-, 1-, 10-, and 100-fold the EC50 calculated in *P. falciparum in vivo* growth inhibition assay, except where upper concentration was limited by solubility, indicated by *.

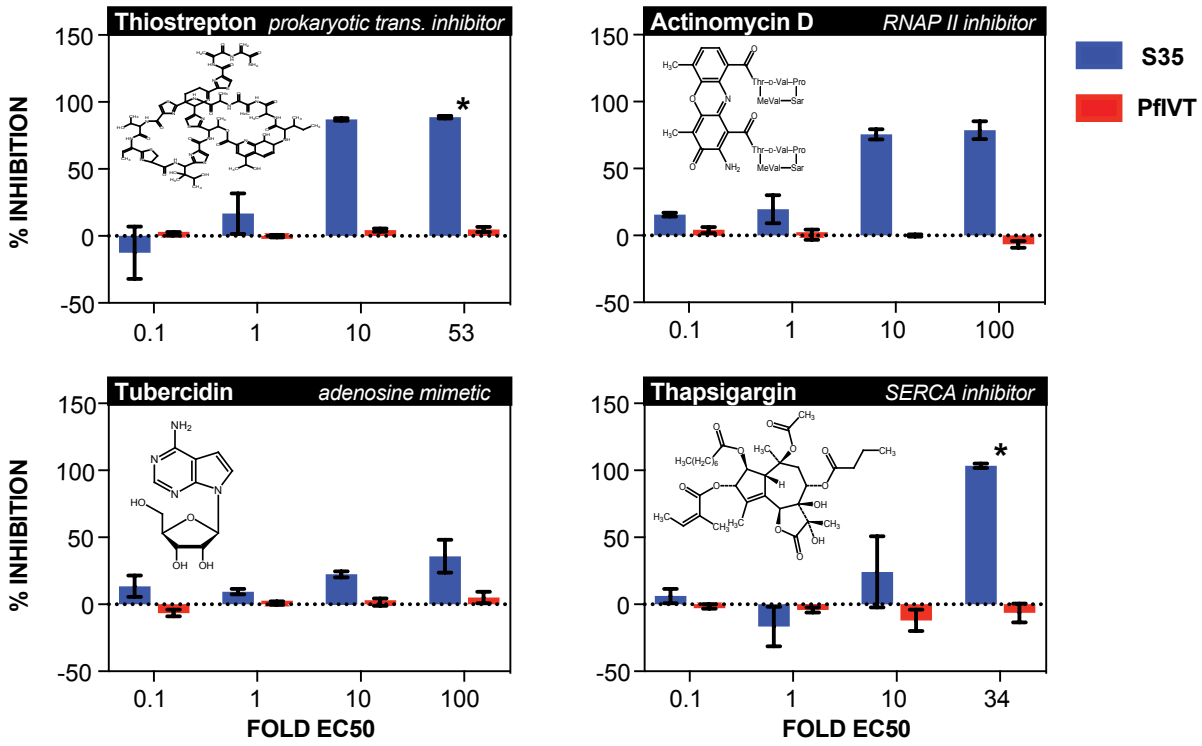

FIGURE 3.2 Dose-dependent inhibition of S35 incorporation and PfIVT by negative control tool compounds.

Dose-dependent inhibition, calculated as % inhibition, of S35 incorporation (blue bars) and PfIVT assays (red bars) by negative control compounds: prokaryotic translation inhibitor and inhibitors of other (non-translation) cellular processes. Name of compound, mechanism of action, and molecular structure are displayed at top of each graph. Compounds were tested at 0.1-, 1-, 10-, and 100-fold the calculated EC50 calculated in *P. falciparum in vivo* growth inhibition assay, except where upper concentration was limited by solubility, indicated by *.

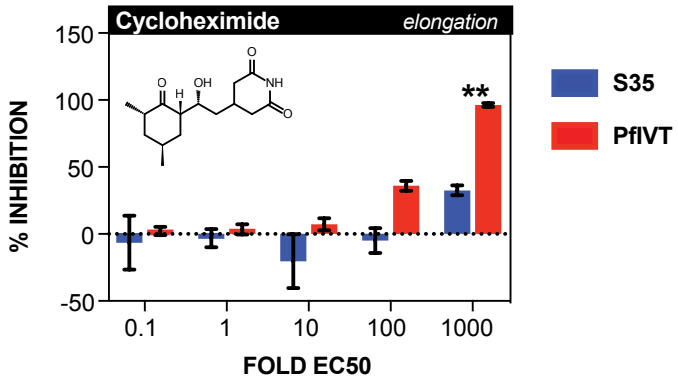

FIGURE 3.3 Dose-dependent inhibition of S35 incorporation and PfIVT by the eukaryotic translation inhibitor cycloheximide.

Dose-dependent inhibition of S35 incorporation (blue bars) and PfIVT assays (red bars) by the translation inhibitor cycloheximide, tested up to 1000-fold (**) the EC50 calculated in *P. falciparum* growth inhibition assay.

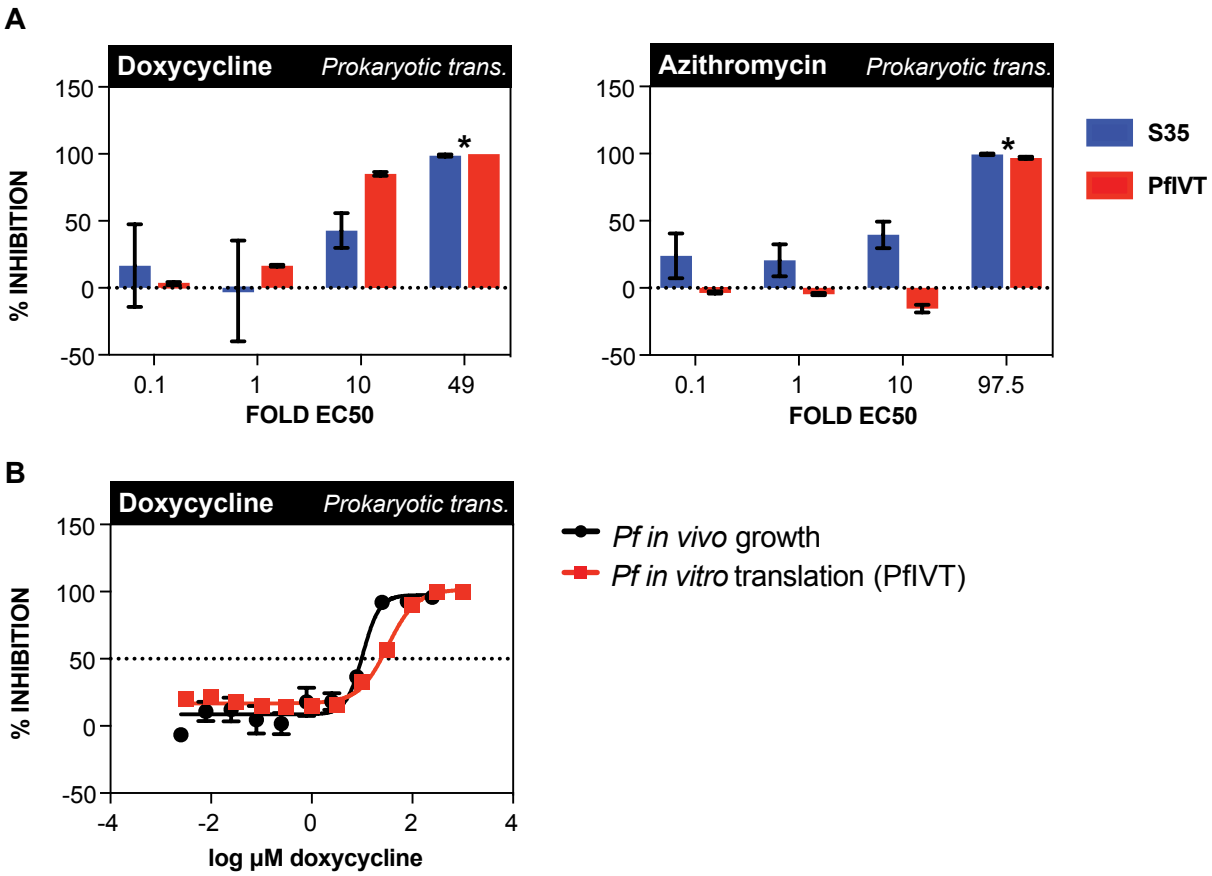

FIGURE 3.4 Dose-dependent inhibition by prokaryote-specific translation inhibitors.

(A) Inhibition of S35-incorporation (blue bars) and PfIVT (red bars) assays by doxycycline and azithromycin, tested at 0.1-, 1-, 10-fold their respective EC50s (indicated on the graph) and the highest concentration achievable given drug stock (*) .
(B) Inhibition of *P. falciparum in vivo* growth (black) and PfIVT (red) assays over serial dilution of doxycycline.

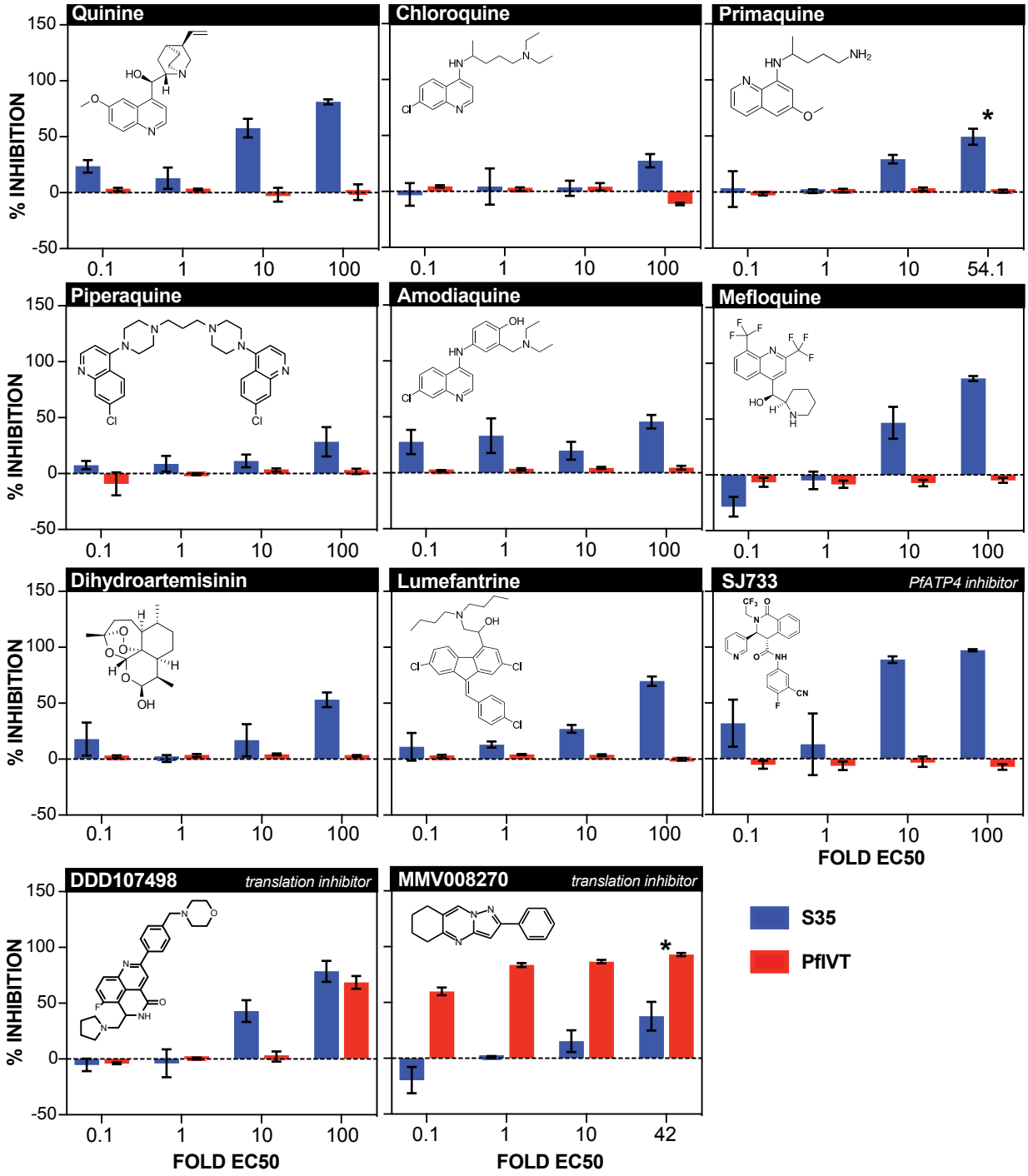

FIGURE 3.5 Dose-dependent inhibition of S35 incorporation and PfIVT by antimalarials.

Dose-dependent inhibition, calculated as % inhibition, of S35 incorporation (blue bars) and PfIVT assays (red bars) by pre-clinical and clinically-approved antimalarial compounds. Name of compound, mechanism of action (where definitively known), and molecular structure are displayed at top of each graph. Compounds were tested at 0.1-, 1-, 10-, and 100-fold the calculated EC50 calculated in *P. falciparum in vivo* growth inhibition assay, except where upper concentration was limited by solubility, indicated by *.

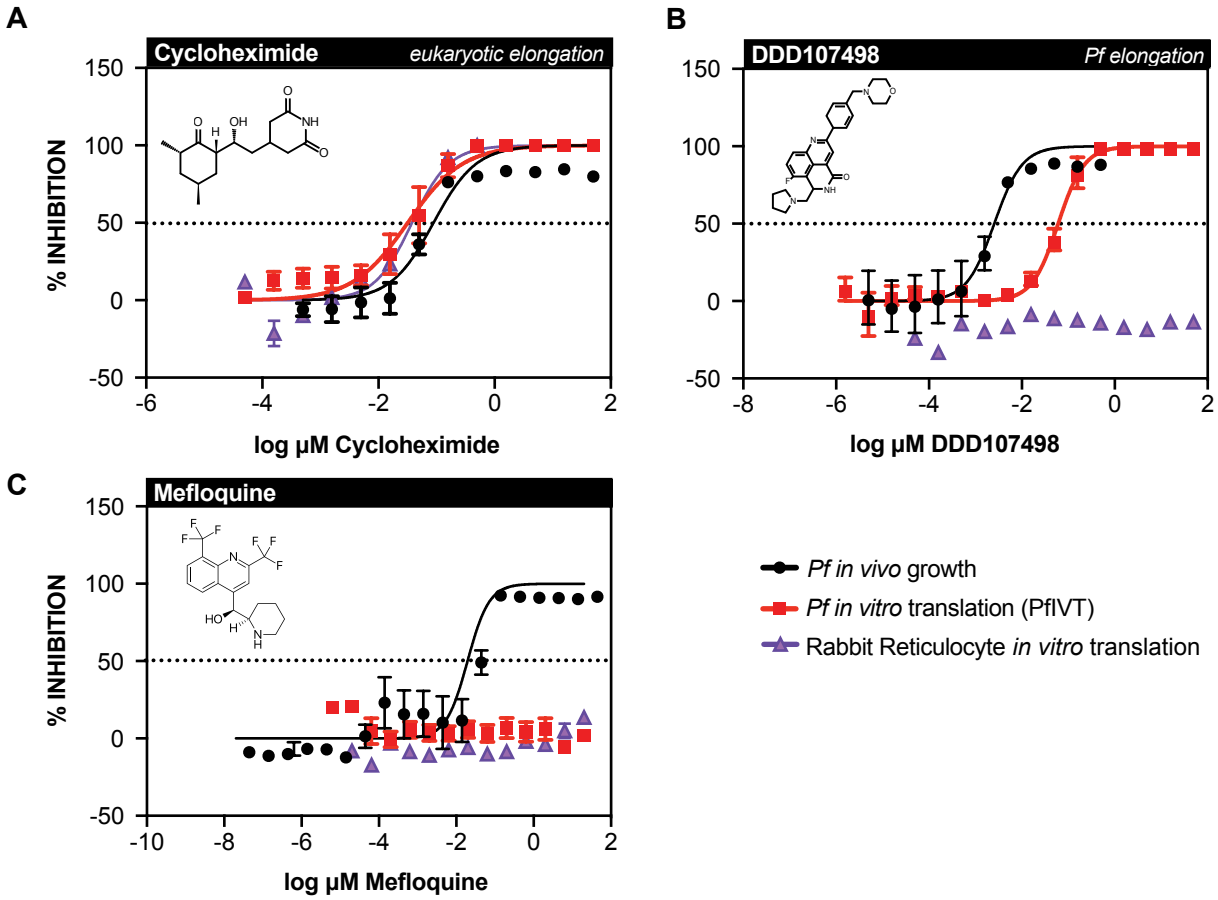

FIGURE 3.6 Dose-response curves of Pf growth, PfIVT, and RRIVT for mefloquine and controls.

Dose-response curves comparing inhibition, calculated as % inhibition, of *P. falciparum in vivo* growth assay (black), *P. falciparum in vitro* translation assay (red), and commercially available rabbit reticulocyte *in vitro* translation assay (purple). Name of compound, mechanism of action (where definitively known), and molecular structure are displayed at top of each graph.

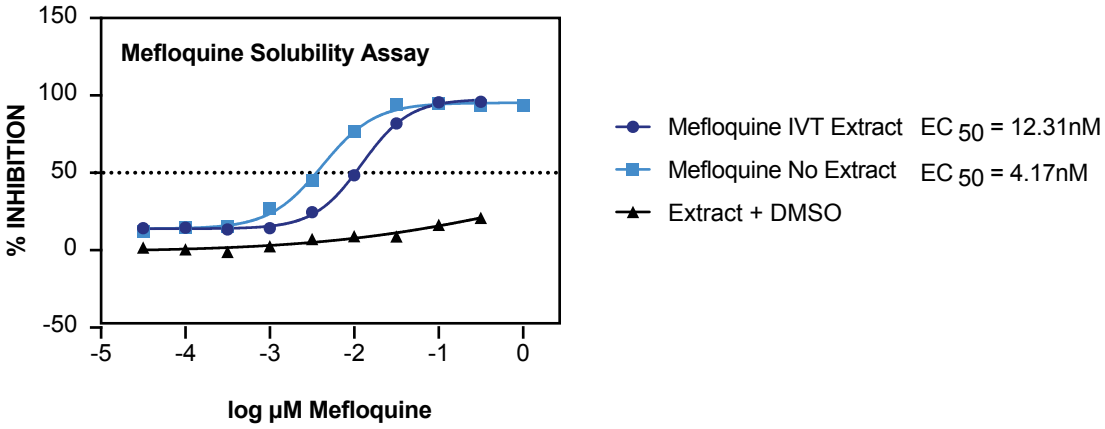

FIGURE 3.7 Mefloquine solubility assay.

Dose-dependent inhibition of *P. falciparum in vivo* growth by mefloquine in PfIVT extract post-PfIVT reaction (Mefloquine IVT Extract), mefloquine alone (Mefloquine No Extract), or DMSO control in PfIVT extract post-PfIVT reaction (Extract + DMSO).

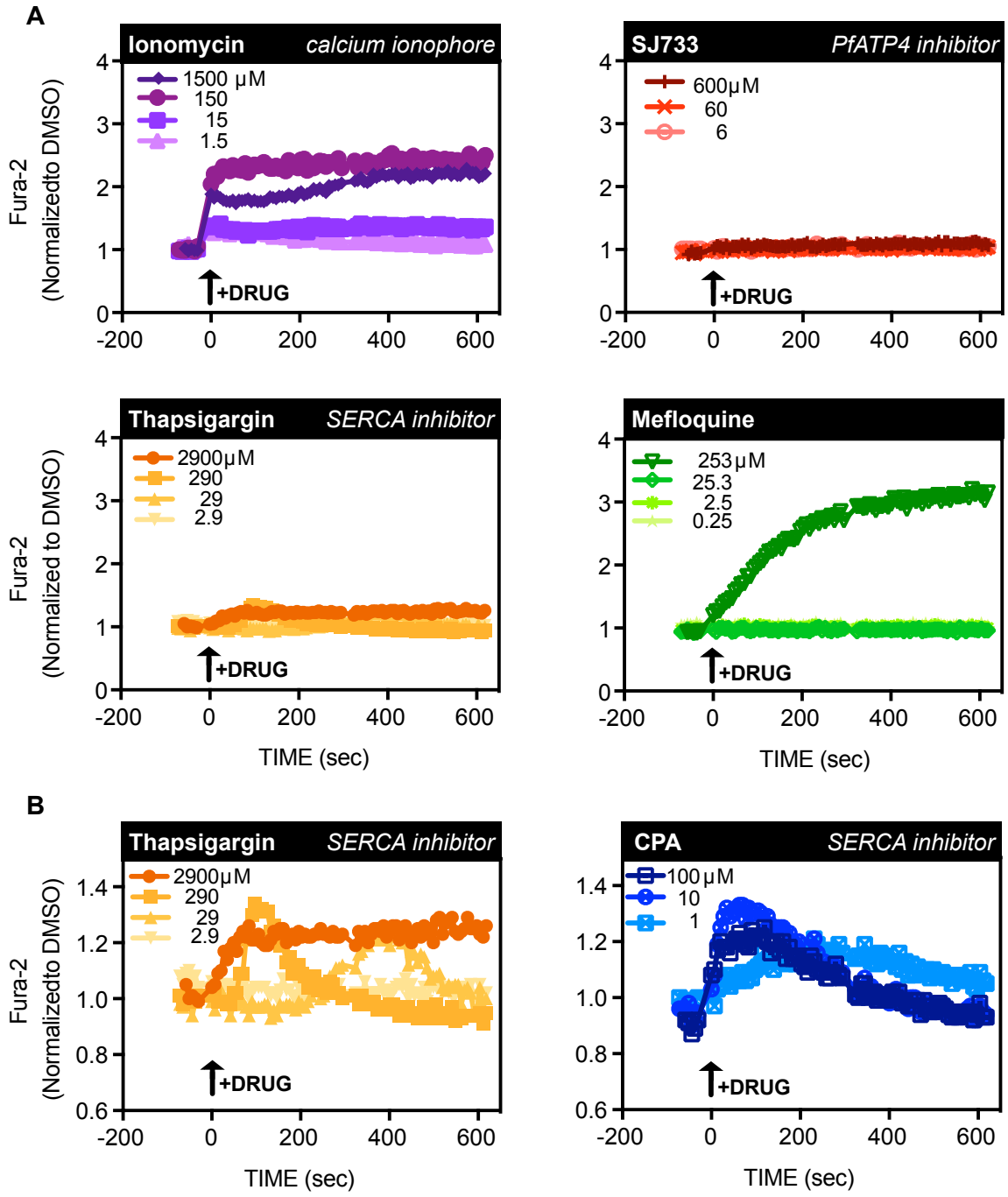

FIGURE 3.8 Addition of mefloquine at high concentrations increases cytosolic calcium levels through emptying of intracellular calcium reservoirs.

Kinetic monitoring of cytoplasmic calcium with addition of drug (at time 0, indicated with arrow), using Fura-2 ratiometric calcium indicator dye. Fura-2 signal normalized to DMSO control. **(A)** Ionomycin (purple), SJ733 (red), thapsigargin (orange), and mefloquine (green). **(B)** Zoomed-in graphs for thapsigargin (orange) and cyclopiazonic acid (CPA; blue).

3.6 TABLES

TABLE 3.1 Mechanism of action and species specificity of translation inhibitors.

COMPOUND	SPECIFICITY	MECHANISM OF ACTION	REFERENCE
bruceantin	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits initiation	(34)
verrucarin A	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits initiation, binds between P- & A- sites	(31–33)
suramin	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits initiation & elongation, multiple binding sites	(35)
anisomycin	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits elongation, binds A-site	(31,36)
cycloheximide	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits elongation, binds E-site	(31)
homoharringtonine	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits elongation on re-initiating ribosomes, binds A-site	(31,37)
lactimidomycin	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits elongation, binds E-site	(31)
nagilactone C	eukaryotic (*Pf* cytoplasmic ribosomes)	inhibits elongation, binds A-site	(31,38)
puromycin	pan-inhibitor	tRNA mimetic	(40,41)
halofuginone	eukaryotic	inhibits glutamyl-prolyl-tRNA synthetase	(39)
thiostrepton	prokaryotic (*Pf* mitochondrial & apicoplast ribosomes)	inhibits initiation & elongation	(42–48)
azithromycin	prokaryotic (*Pf* mitochondrial & apicoplast ribosomes)	inhibits elongation	(52,76)
doxycycline	prokaryotic (*Pf* mitochondrial & apicoplast ribosomes)	inhibits initiation	(53,54)

TABLE 3.2 Mechanisms of action of non-translational inhibitors

COMPOUND	MECHANISM OF ACTION	REFERENCE
actinomycin D	RNA polymerase II inhibitor	(49)
tubercidin	adenosine mimetic	(50)
thapsigargin	SERCA inhibitor	(51,55,56)

TABLE 3.3 Half-maximal effective concentrations (nM) determined in *P. falciparum* growth inhibition assay.

[a,b] Traditional EC50s for these compounds would be determined after 2 growth cycles
[c] Active metabolite of amodiaquine

TEST COMPOUNDS	72H GROWTH INHIBITION EC50 (nM)	ANTIMALARIALS	72H GROWTH INHIBITION EC50 (nM)
bruceantin	4.2	MMV008270	2400
verrucarin A	0.6	SJ733	60
suramin	1819	M5717 (DDD107498)	1
anisomycin	39	quinine	370
cycloheximide	0.6	chloroquine	333
homoharringtonine	6.8	mefloquine	25
lactimidomycin	22	piperaquine	26
nagilactone C	1310	primaquine	1849
puromycin	52	monodesethyl amodiaquine[c]	61
halofuginone	2	lumefantrine	4
thiostrepton	942	dihydroartemisinin	3
azithromycin	13380[a]		
doxycycline	10316[b]		
actinomycin D	10		
tubercidin	168		
thapsigargin	2900		

TABLE 3.4 Mechanism of action and clinical status of antimalarial compounds.

[a] active metabolite of approved drug amodiaquine

COMPOUND	CLINICAL STATUS	MECHANISM OF ACTION & REFERENCE
MMV008270	SCREEN HIT	translation inhibitor (8)
SJ733	CLINICAL TRIALS	cation ATPase PfATP4 inhibitor (26)
M5717 (DDD107498)	CLINICAL TRIALS	translation elongation (PfEF2) inhibitor (7)
quinine	APPROVED	
chloroquine	APPROVED	
mefloquine	APPROVED	
piperaquine	APPROVED	
primaquine	APPROVED	
monodesethyl amodiaquine[a]	APPROVED	
lumefantrine	APPROVED	
dihydroartemisinin	APPROVED	

CHAPTER 4. TRANSLATIONAL REGULATION OF VAR2CSA IN PLACENTAL MALARIA

4.1 INTRODUCTION

P. falciparum infections of the placenta constitute a severe global health burden. Pregnant women (and those who have very recently given birth) are more susceptible to malaria infection and are more likely to develop severe infection than other healthy adults, particularly during their first pregnancy (2,3). In pregnant women infected with *P. falciparum*, the parasite-infected red blood cells (iRBCs) may sequester on the maternal surface of the placenta, in a condition known as placental malaria. Placental malaria infection may result in maternal anemia, maternal mortality, prenatal and neonatal infant mortality, and prematurity and low birth weight, both of which can have very negative consequences for infant health, including increased risk of mortality and other poor outcomes in the first years of life (2,77). Strikingly, iRBCs may sequester so efficiently on the placenta that the mother may show no outward signs of illness, and even pass diagnostic tests, and therefore may not receive appropriate antimalarial therapy (2,16).

The placenta presents an ideal niche for the *P. falciparum* parasite: it is rich in nutrients intended for the developing fetus, relatively immune-privileged to prevent immune rejection of the “foreign” genetic material of the fetus, and the highly efficient transfer of oxygen from maternal to fetal circulation yields a lower oxygen tension at the maternal surface of the placenta relative to other areas of maternal circulation, which is optimal for *P. falciparum* growth (13,14). The parasite has therefore developed a unique mechanism for quickly taking advantage of finding itself in a pregnant host. The

parasite expresses the protein VAR2CSA, which it exports to the surface of the host red blood cell, facilitating sequestration of the iRBC on the placenta through binding to various placental moieties (15,78). Expression of this protein is translationally regulated, allowing for rapid induction of VAR2CSA expression; transcripts are present in all parasites, but the protein is only synthesized when the parasite is in a pregnant host (10,18,19,21,22).

However, little is known about the mechanisms of this translational regulation of VAR2CSA, and how the parasite senses that its host is pregnant. Here, we seek to elucidate the parasite translational response to pregnancy, identifying both *cis*- and *trans*-acting factors contributing to the translational regulation of VAR2CSA. It is our hope that better understanding of this unique biology will provide insight into potential therapeutic targets to prevent placental malaria infection, and the associated devastating outcomes on infant health and mortality.

4.2 RESULTS

4.2.1 VAR2CSA Expression is Repressed at the Level of Translation

Ribosome profiling data from blood stage cultures of the *P. falciparum* strain W2 demonstrate that expression of VAR2CSA is translationally repressed (22). These data further suggest that the repression is occurring, at least in part, as a result of accumulation of ribosomes on the 5'UTR of the *var2csa* transcript. Through use of the PfIVT assay, we sought to determine whether the VAR2CSA 5'UTR is sufficient to

repress translation *in vitro.* We utilized firefly luciferase constructs containing the 3'UTR from the *P. falciparum* histidine rich protein (PfHRP) and the 5'UTR from either VAR2CSA or the ubiquitously expressed erythrocyte binding antigen (EBA-175) as a non-translationally regulated control (FIGURES 2.3 & 4.1). We found that the presence of the VAR2CSA 5'UTR represses translation, resulting in luciferase signal approximately 50-fold lower than that produced by the construct containing the EBA-175 5'UTR (FIGURE 4.2). In contrast, comparison of these two constructs in the commercially available RRIVT assay demonstrates no difference in translation between the two, suggesting that the repressive function of the VAR2CSA 5'UTR is specific to *Plasmodium* (FIGURE 4.2).

4.2.2 Putative uORF Sequence, But Not Translation, Contributes to VAR2CSA Repression

The two predominant ribosome peaks on the 5'UTR of VAR2CSA are found near a putative uORF previously described in the literature (19,22). To determine whether the uORF plays a role in the translational repression of VAR2CSA, we generated a construct with the putative uORF deleted, and one in which the putative uORF has been replaced by a scramble (FIGURE 4.1). Both constructs resulted in a significant increase of translation in the PfIVT assay as compared to wild type, demonstrating that the putative uORF contributes to the repression of VAR2CSA translation in a sequence-specific manner (FIGURE 4.3). We further mutated the start codon of the putative

uORF to determine whether it exhibits canonical uORF inhibition of translation by being translated itself (FIGURE 4.1). We found that, contrary to previous literature utilizing an indirect measure of translation, the ablation of the start codon had no effect on translation relative to wild type, demonstrating that the "uORF" exerts its inhibitory function through a mechanism other than the canonical model of uORF-mediated repression (FIGURE 4.3) (19).

Given the presence of a ribosomal peak centered approximately 100 bases upstream of the putative "uORF," we questioned whether this upstream region contributes to repression of VAR2CSA translation. Deletion of this region resulted in an increase in translation in the PfIVT assay equivalent to deletion of the similarly-sized "uORF," suggesting that this portion of the VAR2CSA 5'UTR also plays a regulatory role in repressing VAR2CSA translation (FIGURES 4.1 & 4.4).

4.2.3 Identification of a Novel Regulatory Element Contributing to Repression of VAR2CSA Translation

Because of its robust growth in laboratory culture and amenability to maintenance at high parasitemia, we utilized the W2 strain of *P. falciparum* for our PfIVT assays, rather than the 3D7 strain utilized for previous VAR2CSA studies. As a result, we identified an insertion of 233 bases in length downstream of the "uORF" in the 5'UTR of W2 *var2csa* that had not been previously described. Deletion or scrambling of this insertion, which we have denoted the ARE (Additional Repressive Element), yields a greater than 2-fold increase in translation relative to the VAR2CSA wild type 5'UTR,

when utilizing a nanoluciferase reporter (which yields a smaller dynamic range than the firefly reporter in these assays) (FIGURE 4.5). Additionally, the 3D7 wild type VAR2CSA 5'UTR is significantly less translationally repressive than the W2 wild type VAR2CSA 5'UTR, despite having nearly 98% similarity outside of the ARE (FIGURE 4.6A). Together, these data suggest that the ARE act as an additional inhibitor of translation, also in a sequence-specific manner.

Presence of start and stop codons near the open reading frame (ORF) can affect translational efficiency (77). The ARE introduces a stop codon (145 bases downstream of the uORF) to the W2 VAR2CSA 5'UTR that is not present in the 3D7 VAR2CSA 5'UTR, in addition to another stop nearby stop codon found in W2 but not 3D7 (88 bases downstream of the uORF). Insertion of just these stop codons (in the absence of the ARE) to the 3D7 VAR2CSA 5'UTR, using a nanoluciferase reporter, decreases translation in the PfIVT assay (FIGURES 4.1 & 4.5B). Together with the observation that deletion of these stop codons from the W2 VAR2CSA 5'UTR (again, using a nanoluciferase reporter), without other changes to the ARE, increases translation in the PfIVT assay, these data suggest that part of the translational repression exerted by the ARE may result from the additional stop codon (FIGURES 4.1 & 4.5B).

4.2.4 Geographic Distribution of the ARE

The VAR2CSA 5'UTR from an additional *P. falciparum* strain, ACPGFP, was also determined to contain the ARE. Interestingly, both W2 and ACPGFP, the strains containing the ARE, are of Southeast Asian origin, while 3D7 is of African origin (79–

82). In addition, the VAR2CSA 5'UTRs of both W2 and ACPGFP are considerably more translationally repressive than that of 3D7 in the PfIVT assay (FIGURE 4.6A). To determine whether the ARE is naturally occurring, or arose as an artifact of decades of laboratory culture, we assembled the VAR2CSA 5'UTRs from publicly-available sequences from 224 *P. falciparum* primary patient samples from the Wellcome Sanger Institute, collected from 6 countries in African and 6 countries in Southeast Asia. We identified the ARE in 14 patient samples, with a prevalence of 12% in the Southeast Asian samples, compared with a prevalence of only 2% in the African samples (FIGURE 4.6B). Interestingly, in a phylogenetic tree constructed with only the VAR2CSA 5'UTRs from these samples, the samples are distributed primarily according to geographic origin, with one large branch consisting of exclusively Asian isolates, and another distinct branch, most closely related to the Asian branch, consisting only of the 14 samples containing the ARE (FIGURE 4.6C).

4.2.5 Maternal Serum from First and Second Trimesters of Pregnancy Stimulates VAR2CSA Translation

To address the question of what the *P. falciparum* parasite is sensing in a pregnant host, inducing it to turn on VAR2CSA translation, we added serum obtained from a woman (Donor B) in the 2nd trimester of her pregnancy to the PfIVT and RRIVT assays, comparing firefly luciferase constructs with either the W2 wild type VAR2CSA 5'UTR or the EBA-175 control. Serum from a healthy male was also tested as a control in the assay. A consistent and reproducible increase in translation of the VAR2CSA

construct was observed with addition of Donor B (pregnant) serum, as compared to EBA-175 control or addition of male serum (FIGURE 4.7). Further, this induction of VAR2CSA translation with addition of pregnant serum was specific to *P. falciparum*, and did not occur in the RRIVT assay (FIGURE 4.7).

In accordance with previous reports on addition of serum to *in vitro* translation assays, addition of any serum to either the PfIVT or RRIVT assays was strongly inhibitory (FIGURE 4.8A). We confirmed that the inhibition of the PfIVT assay by serum is the result of inhibition of translation itself, rather than inhibition of luciferase enzyme activity by comparing addition of male serum at the beginning of the PfIVT assay with a 2-hour incubation, and spiking in the same serum for the final 15 minutes of a 2-hour incubation of a PfIVT reaction, finding that the serum spike had no effect on luminescence signal, while addition of serum at the beginning of the assay nearly ablated luminescence signal in the assay (FIGURE 4.8B). To improve the dynamic range of the assay with serum, we were able to remove inhibitory components in the serum with a simple molecular weight cut-off (MWCO) filtration, and found that a 30Kda MWCO filter traps most of the inhibitory factors in the retentate, while the pregnancy-specific translation-inducing factor(s) is preserved in the flow-through (FIGURE 4.8C).

Prior to implementation of the MWCO filtration protocol, induction of translation by pregnant serum could be better observed through comparing activity curves generated through addition of a dilution series of sample to the PfIVT assay (FIGURE 4.9). Generation of these curves with a panel of samples obtained from pregnant women in the 3rd trimester of pregnancy indicated that there is likely a gestational age-dependent effect, as none of the 3rd trimester samples showed any difference in the

PfIVT assay between EBA-175 and VAR2CSA constructs, with respect to male or nonpregnant female controls (FIGURE 4.9). Seeing this result, we chose to focus on samples from the 1st and 2nd trimesters of pregnancy for further study. We prepared a panel of samples consisting of serum samples from 16 males, 16 nonpregnant females, and 16 pregnant females in the 1st or 2nd trimesters of pregnancy. After MWCO filtration, sera were added to the PfIVT assay, and the relative effects on EBA-175 and VAR2CSA constructs were assessed for each sample. Although there is substantial variation within each group, the pregnant sera specifically induce translation of the VAR2CSA constructs more than the nonpregnant female sera ($P = 0.0003$) and the male sera ($P < 0.0001$) (FIGURE 4.10). It is likely that some variation within the pregnant sera group is due to differences in gestational age or gravidity; as such, further study of a larger number of samples from all stages of pregnancy, in women who are in their first pregnancy and in women who have had multiple pregnancies is warranted. Additionally, hormonal birth control status of the nonpregnant female controls was not collected, which may contribute to some of the variation within the control group; follow-up studies comparing a larger group of nonpregnant females who use hormonal birth control verses those who don't may provide useful information. Most strikingly, while the 2nd trimester sample from Donor B yielded the strongest VAR2CSA-specific effect of any sample tested, a sample collected from the same donor 1 year post-partum had negligible effect on induction of VAR2CSA translation, demonstrating that the effect was a result of pregnancy, rather than simply a characteristic of unique to Donor B herself (FIGURE 4.10).

4.2.6 Increased pH Resulting from Placental Enzymes May Induce Translation of VAR2CSA

To identify the factor(s) in pregnant sera that induce translation of VAR2CSA, we performed mass spectrometry on the 2nd trimester sample from Donor B. We identified two candidate proteins, lactate dehydrogenase A chain (LDHA), and carbonic anhydrase I (CA1) (TABLE 4.1). Production of both enzymes is increased during pregnancy, and both are found in circulation, within erythrocytes, and on the placenta (83–88). Addition of either enzyme to the PfIVT assay had no effect on translation, but these experiments may require significant optimization to obtain reliable results.

One effect of both enzymes at the maternal surface of the placenta is likely an increase in pH of the local microenvironment. To test whether this increase contributes to the induction of VAR2CSA translation in pregnant hosts, we performed the PfIVT and RRIVT assays with both VAR2CSA and EBA-175 control reporter constructs across a range of pH, through increasing the HEPES buffer by 10-fold in the 10X Translation Mixes, and adding HEPES of different pH, in addition to the standard pH7.5 HEPES. pH of the reactions was measured via microprobe at 37°C just prior to transfer to the luminescence assay plates and subsequent addition of luciferin reagent. The highest pH that we were able to achieve using a HEPES buffer system (as determined by reaction endpoint measurement) was pH 7.69; the lowest was pH 7.01. Surprisingly, there appears to be a global increase in *P. falciparum* translation, based on the EBA-175 control reporter, with increasing pH, although it seems to plateau at the two highest pH conditions tested (FIGURE 4.12). In contrast, rabbit reticulocyte translation levels

are highest at baseline pH, with modest decreases in activity as pH deviates from baseline (FIGURE 4.12). Most interestingly, in the PfIVT assay, translation specifically of the VAR2CSA reporter increases even more than the general increase in translation seen with increasing pH, and does not appear to have reached its maximum level at the pH achievable with HEPES buffering in this assay (FIGURE 4.12). Notably, this effect is also specific to PfIVT and is not seen in RRIVT, where the VAR2CSA and EBA-175 control reporters have comparable activities (FIGURE 4.12).

Additionally, in a small subset of serum samples from pregnant and nonpregnant individuals, there is a slight positive correlation between increasing reaction endpoint pH and specific increases in VAR2CSA translation (FIGURE 4.13). However, this correlation is currently weak and requires validation across a much larger sample set.

4.2.7 Putative uORF, But Not ARE, Contributes to pH/Pregnancy Response of VAR2CSA

We next sought to ascertain whether the regulatory elements within the 5'UTR that we determined to contribute to inhibition of VAR2CSA translation also participate in the induction of VAR2CSA translation in the setting of pregnancy. To this end, we tested reporter constructs with the control EBA-175 5'UTR, the wild type VAR2CSA 5'UTR, and the VAR2CSA 5'UTR with the putative uORF substituted with the positionally-corresponding segment of the EBA-175 5'UTR, at baseline pH, and at the highest pH achievable with HEPES buffer in the assay (pH 7.69). The wild type VAR2CSA 5'UTR reporter exhibited a greater change in translation with increasing pH

than both the control EBA-175 5'UTR reporter and the VAR2CSA substituted uORF 5'UTR reporter, which were equivalent to each other (FIGURE 4.14A). These results suggest that the putative uORF is at least in part responsible for the VAR2CSA-specific increase in translation with increasing pH.

To determine what role, if any, the ARE plays in the response to pregnancy, we compared the wild type VAR2CSA 5'UTR reporter constructs from the W2 and 3D7 strains with and without addition of pregnant serum from Donor B. Relative to no serum addition, the percent increase in VAR2CSA translation with addition of pregnant serum was approximately the same in both constructs (FIGURE 4.14B). Given that the VAR2CSA 5'UTRs from the two strains are nearly 98% identical, except for the presence (in W2) or absence (in 3D7) of the ARE, it is unlikely that the ARE is involved in the serum response.

4.3 DISCUSSION

In this work, we confirm that VAR2CSA is regulated at the level of translation, and is translationally repressed under "normal" conditions (i.e. when in a nonpregnant host), using the *P. falciparum in vitro* translation (PfIVT) assay. Importantly, by utilizing a direct measure of translation, rather than the indirect methods previously used to study VAR2CSA regulation, we were able to demonstrate identify sequence elements within the 5'UTR of VAR2CSA that contribute to the translational repression of the gene. Further, through study of serum samples from pregnant women in this PfIVT assay, we were able to demonstrate changes in serum composition during pregnancy contribute,

at least in part, to the de-repression of translation of VAR2CSA in the setting of pregnancy. Interestingly, study of the serum samples additionally demonstrated that the different regulatory sequence elements within the 5'UTR contribute to repression of VAR2CSA via different mechanisms. Together, these data bring us closer to an understanding of how the parasite is able to respond specifically to pregnancy, turning on expression of the VAR2CSA protein, and taking advantage of the placental niche.

We identified two elements within the VAR2CSA 5'UTR that contribute to repression of VAR2CSA translation: the putative "uORF," previously described in the literature, as well as a novel regulatory element, the Additional Repressive Element, or ARE. We confirmed that the previously annotated "uORF" does repress translation of VAR2CSA in a sequence-specific manner. However, prior reports relied upon transient transfection of drug resistance reporter plasmids and are thus an indirect measure of translation, and the observed changes in production of the reporter may actually be the effects of other cellular processes. In contrast to these reports, we found that the "uORF" does not, in fact act as a canonically repressive uORF, in that it does not require translation itself to exert its repressive function; ablation of the start codon had no effect in the PfIVT assay. The PfIVT results, combined with ribosome profiling data revealing ribosomal peaks further upstream of the "uORF," suggest that the repressive element is likely not the "uORF" itself, but rather that there is a repressive element beginning upstream, and then extending into and including a portion of the "uORF." Further dissection of the VAR2CSA 5'UTR and study of this region in particular are needed to better elucidate the repression of VAR2CSA translation.

We also identified a separate repressive element in the VAR2CSA 5'UTR: the novel Additional Repressive Element, or ARE. This element was likely not identified previously, due to use of the 3D7 laboratory strain in prior VAR2CSA studies. We found the ARE to be present in the 5'UTR of the W2 and ACP-GFP laboratory strains (which are of Asian origin), but not the 3D7 strain (of African origin). We confirmed that the ARE is not an artifact of laboratory culture, but is, in fact, present in primary patient isolates. Most interestingly, we found that the ARE, which contributes an added level of VAR2CSA translational repression in a sequence-specific manner, is present at much higher rates in parasite samples from Southeast Asia (14 of 118 samples) than in parasite samples from Africa (2 of 106). As migration of parasites from Southeast Asia to Africa has been well documented, there is the possibility that 2 samples in the African cohort containing the ARE are not actually of African origin, and we are currently working to determine the geographic origin of these samples. Importantly, the VAR2CSA 5'UTR, and the ARE in particular, appears to be highly geographically-selected, as the VAR2CSA 5'UTR produces geographic structure in a phylogenetic tree, producing a branch consisting of only the ARE-containing samples, and a closely-related branch consisting exclusively of Asian samples. This stands in stark contrast to the lack of geographic and phylogenetic structure of the VAR2CSA ORF itself, as reported in the literature (89,90). Additionally, we have found the VAR2CSA 5'UTR to be highly conserved (~97% identity excluding the ARE, ~96% including the ARE, and 100% identify of the ARE itself) among patient samples, while the VAR2CSA ORF is known to be highly polymorphic, further highlighting the importance of the regulatory role of the VAR2CSA 5'UTR, and the likely geographic selection of the ARE (89,90).

Most interestingly, the relative geographic prevalence of the ARE correlates with the differences in prevalence of placental malaria; in Southeast Asia, where the ARE is more prevalent (and should, in theory, create a higher barrier to expression of VAR2CSA), rates of placental malaria are lower as compared to Africa, where the ARE is less prevalent (2,3,77,91–94). There are likely several contributing factors to this difference in prevalence of placental malaria, such as proportion of *P. falciparum* vs. *P. vivax* cases, differences in patterns and frequency of transmission, however, it is possible that the ARE also plays a role.

In addition to identifying cis-acting elements within the VAR2CSA 5'UTR that contribute to its regulation, we further determined that the parasite is responsive to a factor, or factors, present in the maternal circulation during the early stages of pregnancy, when the placenta is undergoing rapid development. While we have mass spectrometry data to suggest that the VAR2CSA-inducing factor is either LDHA or CA1, both of which are produced by placenta, we are working to confirm this. However, we have determined that the parasite is likely responding to localized increases in pH on the maternal surface of the placenta, as our data demonstrate that increases in pH result in a specific increase in VAR2CSA translation. Importantly, our data suggest that the repressive element encompassing the putative "uORF" contributes to this pH response, while the ARE functions via a separate, pH-independent mechanism.

Together, this work suggests a model of VAR2CSA translational regulation in which the default "mode" for VAR2CSA translation is repression, but in *P. falciparum* parasites infecting women in the first or second trimesters of pregnancy, which are exposed to increased pH in the maternal circulation near the placenta, VAR2CSA

translation is induced via a yet-to-be-determined mechanism involving a cis-regulatory element encompassing the putative "uORF." Expression of VAR2CSA then allows parasites to establish a placental infection. Further, in parasites possessing an additional cis-regulatory factor, the ARE, present at higher rates in parasites of Asian origin than African origin, the repression of VAR2CSA is even stronger; therefore, VAR2CSA in pregnant women infected with parasites lacking the ARE is likely expressed at higher levels, making the parasites even more likely to infect the placenta. It is our hope that this study lays the foundation for a better understanding of this unique biology, revealing potential therapeutic targets to be exploited to prevent placental malaria infections.

4.4 MATERIALS AND METHODS

Ethics Statement

This study was approved by the University of California, San Francisco Committee on Human Research. Written informed consent was obtained from all participants.

Serum collection and MWCO Filtration

Serum samples from pregnant women were provided by Dr. Laura Jeliffe-Pawlowski of UCSF. Control samples were collected from healthy male and female donors. 10mL blood was collected into additive-free serum blood collection tubes (BD #366430), and allowed to clot for 1-2 hours at room temperature. Tubes were then centrifuged at 1200x*g* for 20 minutes at room temperature. Serum was carefully removed and

aliquoted into tubes, flash-frozen in liquid nitrogen, and stored at -80ºC. One aliquot remained on ice (rather than being frozen) for immediate filtration, as described below.

To remove inhibitory factors from serum for use in PfIVT Assay, serum was filtered using a 0.5mL 30KDa centrifugal filter (EMD Millipore #UFC503096). Filters were pre-chilled on ice, then washed with 500µL Buffer B2, centrifuged for 6 minutes at 14,000x*g* and 4ºC. Buffer B2 was carefully removed from collection tube and filter column. 50µL serum was then applied to the washed filter, and centrifuged for 26 minutes at 14,000x*g* and 4ºC. Serum flow-through was carefully retrieved from the collection tube, aliquoted into pre-chilled tubes, flash-frozen in liquid nitrogen, and stored at -80ºC. This flow-through is the portion utilized in the work described in this chapter; retentate was also collected, but was not utilized. Retentate was collected by inverting the filter into a fresh collection tube and centrifuging for 2 minutes at 1,000x*g* and 4ºC, transferred to a fresh tube, flash-frozen, and stored at -80ºC.

Plasmodium falciparum In Vitro Translation Assay

PfIVT assays were carried out as described in detail in Chapter 2, with the following alterations: 1pmol of mRNA reporter transcript was utilized per 20µL reaction, and all mRNA reporter transcripts were electroeluted prior to use. In assays utilizing serum, 1µL of serum was utilized per 20µL reaction. For studies investigating the role of pH, the 10X TM contained 200mM HEPES of either pH6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0 (in contrast to the standard 20mM HEPES pH7.5 in all other PfIVT assays).

Rabbit Reticulocyte In Vitro Translation Assay

RRIVT assays were carried out as described in Chapter 3, Section 3.3.6 (Rabbit Reticulocyte In Vitro Translation Assay), with the following alterations: 1pmol of mRNA reporter transcript was utilized per 20µL reaction, and all mRNA reporter transcripts were electroeluted prior to use. In assays utilizing serum, 1µL of serum was utilized per 20µL reaction. For studies investigating the role of pH, the reaction composition was as follows: 20µL: 7µL reticulocyte lysate, 1pmol T7 transcribed reporter mRNA, 10µM amino acid mixture, 2µL 10X RRTM, and DEPC H_2O to final volume. The 10X RRTM differs from the translation mix provided with the rabbit reticulocyte kit and its composition is as follows: 800mM KOAc, 5mM $Mg(OAc)_2$, 100mM phosphocreatine, and 200mM HEPES of either pH6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0.

Mass Spectrometry

Serum samples analyzed via mass spectrometry included one male control and one second trimester pregnancy sample. Both were 30 KDa MWCO filtered, and only the flow-through was utilized.

Proteomic analysis was performed by liquid chromatography-tandem mass spectrometry, LC-MS/MS. Samples were prepared by in gel digestion with trypsin following the UCSF Mass Spectrometry Facility protocol (http://msf.ucsf.edu/protocols.html). Briefly, gel bands were diced into small cubes, then washed/destained twice with 50:50 acetonitrile (ACN): 25 mM ammonium bicarbonate (ABC) solution. Samples were reduced with 5 mM dithiothreitol in ABC for 30 min at 56 °C, then alkylated with 10 mM iodoacetamide in ABC for 1 h, in the dark at room

temperature. Samples were washed again 2x with 50:50 ACN:ABC, and solvent was removed prior to the addition of trypsin in ABC at 1:50 µg trypsin : µg protein sample for overnight digestion at room temperature. Samples were extracted from the gel pieces with 50:50 ACN: 0.1% formic acid. Peptide extracts were dried under vacuum, then resuspended in 0.1% formic acid for liquid chromatography-tandem mass spectrometry LC-MS/MS analysis.

Peptides were sequenced using an LTQ Orbitrap Velos mass spectrometer (Thermo), coupled to a 10,000 psi nano-Acuity UPLC system (Waters) for reversed phase separation on a C18 EZSpray column (Thermo, 75 µm i.d. x 15 cm, 3 µm bead size, 100 Å pore size). Peptides were separated over 60 min using a linear gradient of 2-30% acetonitrile in 0.1% formic acid at 300 nl/min flow rate. Survey scans were acquired at 30,000 resolution in the FT over a 325-1400 m/z range, followed by higher energy collisional dissociation (HCD) fragmentation scans of the six most intense ions, measured in the Orbitrap using a threshold of 2000 counts, 2.0 m/z isolation width, 30% normalized collision energy, and 30 µs activation time. The polydimethylcyclosiloxane ion with m/z = 445.120025 was used for internal calibration of both precursor and fragmentation scans.

Mass spectrometry peak lists were generated with an in house script called PAVA, and data were searched with UCSF software Protein Prospector, v. 5.19.1 (95). Data were searched with a database of SwissProt human sequences (downloaded December 1, 2015) plus background proteins (BSA, trypsin, and streptavidin), containing 20,194 sequences. A randomized decoy database of 20,194 sequences was used for estimation of false discovery rate (96). Mass accuracy tolerance was 20 ppm

and 30 ppm for the precursor and fragment scans respectively. Data searches allowed up to two missed cleavages, and the following modifications: Carbamidomethylation of Cys was a fixed modification, and variable modifications included oxidized Met, pyroglutamate from N-terminal Gln, start Met processing, acetylation of the N-terminus. Protein and peptide false discovery rates were <1%, with Protein Prospector minimum protein and peptide scores of 22 and 15 respectively, and with maximum expectation values of 0.01 for protein and 0.05 for peptides.

pH measurement

pH measurements of *in vitro* translation assays were taken at the endpoint of the assay, prior to transferring to 96-well plate for luciferase assay. pH was measured at 37°C, using an Orion™ PerpHecT™ ROSS™ Combination pH Micro Electrode (Thermo Fisher #8220BNWP).

Primary Patient Sample Sequences and Assembly

Publicly available whole genome sequencing data from *P. falciparum* primary patient samples were obtained from the Wellcome Sanger Institute's MalariaGEN database at http://malariagen.net/apps/pf3k/release_3/index.html#table_samples We utilized only data meeting the following criteria: read length 100 bases or greater, quality score greater than 30, and mean coverage greater than 100. 224 samples met these criteria: 37 from Ghana, 32 from Malawi, 8 from Democratic Republic of Congo, 20 from Guinea, 3 from Mali, 6 from Senegal, 28 from Cambodia, 43 from Thailand, 24 from Vietnam, 12 from Laos, 8 from Myanmar, and 3 from Bangladesh.Bowtie2 was used to identify

VAR2CSA 5'UTR seeds for assembly from the samples, and VAR2CSA 5'UTR sequences were assembled using PRICE Genome Assembler.

4.5 FIGURES & TABLES

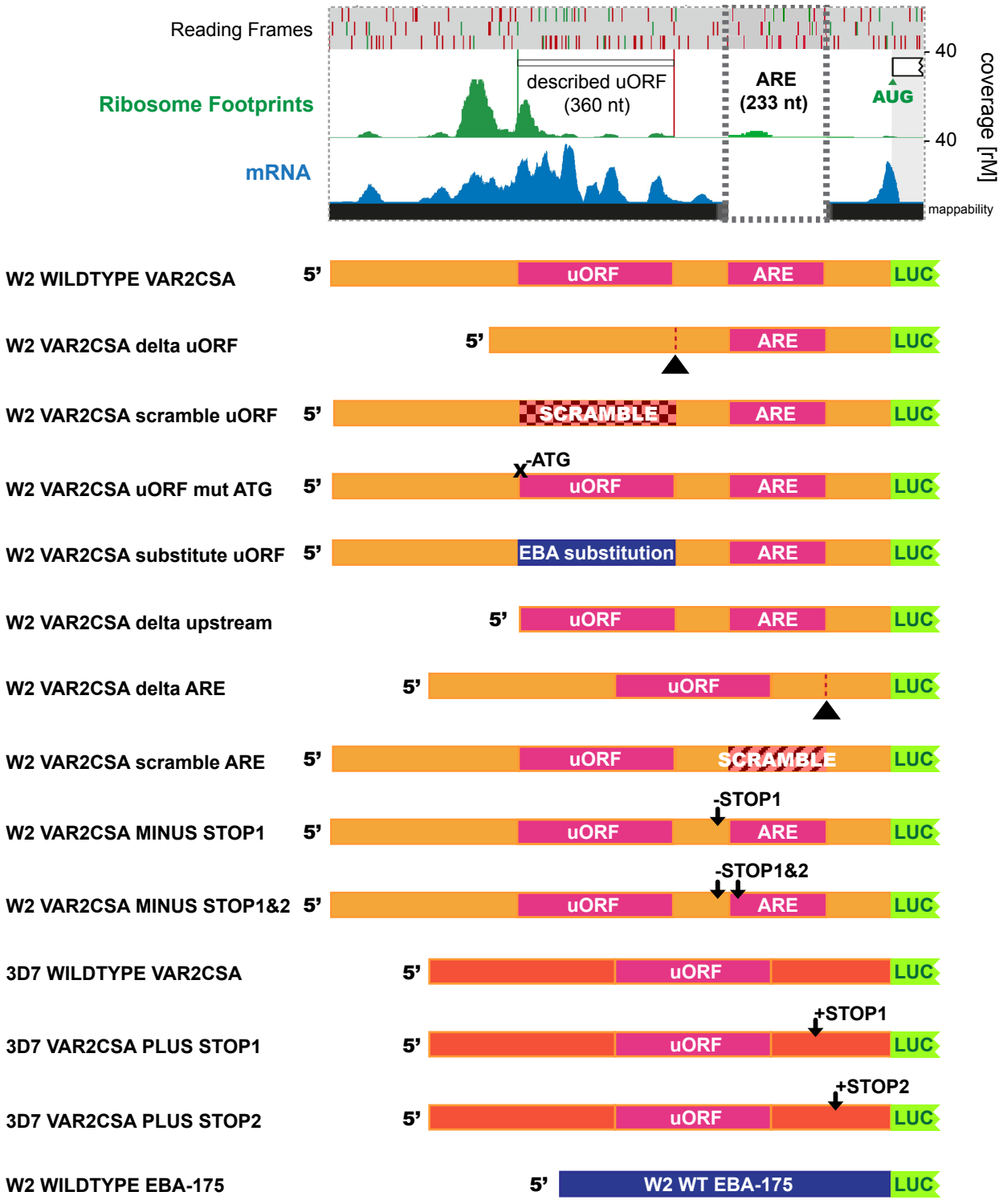

FIGURE 4.1 Diagram of luciferase reporter transcripts used to investigate regulatory function of VAR2CSA 5'UTR. All constructs drawn to scale. W2 WT VAR2CSA aligned to ribosome profiling data. LUC = luciferase ORF

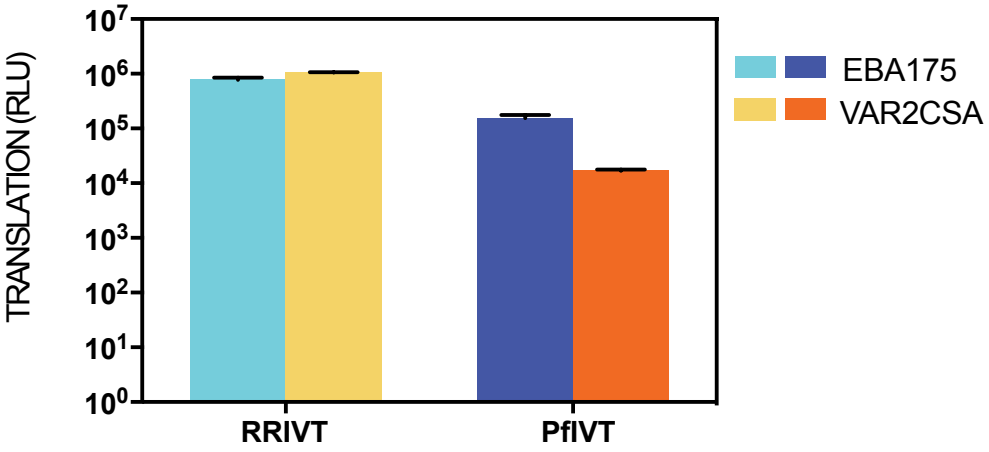

FIGURE 4.2 ***P. falciparum*-specific repression of VAR2CSA translation.**

Comparison of translation of VAR2CSA 5'UTR reporter construct (yellow/orange bars) and EBA175 5'UTR control reporter construct (blue bars) in the commercially available rabbit reticulocyte *in vitro* translation assay and the *P. falciparum in vitro* translation assay.

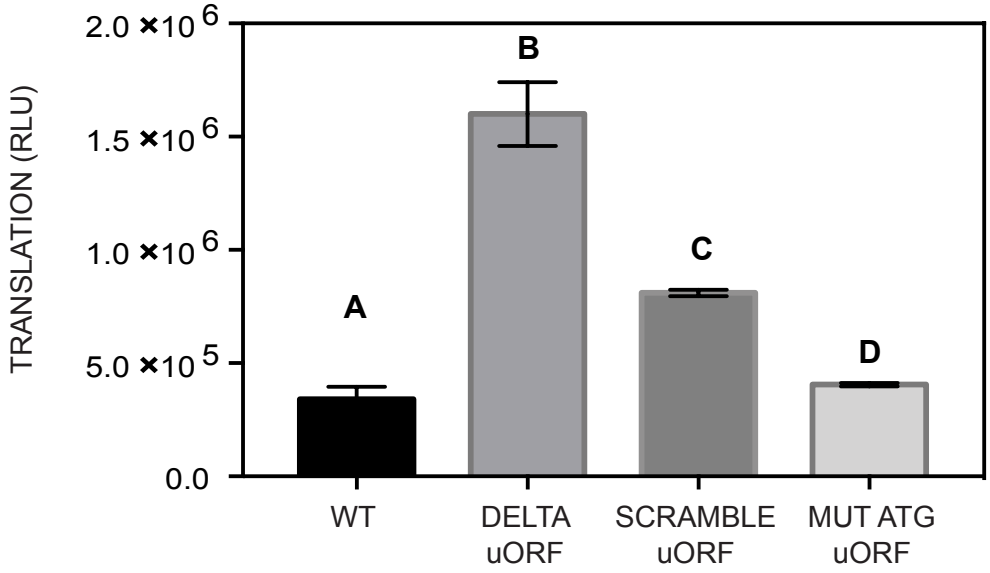

FIGURE 4.3 Putative uORF represses translation of VAR2CSA in non-canonical sequence-dependent manner.

Comparison of PfIVT activity of firefly luciferase reporter constructs reveals that "uORF"-mediated translation repression is dependent upon sequence of the uORF (rather than solely length), but that translation of the uORF is not required for inhibition:
(A) wildtype W2 VAR2CSA 5'UTR
(B) W2 VAR2CSA 5'UTR with uORF deleted
(C) W2 VAR2CSA 5'UTR with scramble uORF
(D) W2 VAR2CSA 5'UTR with ATG mutated to ablate start codon

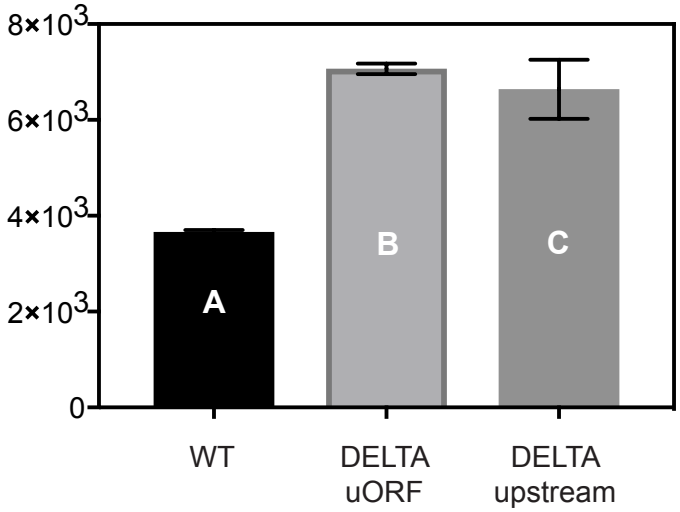

FIGURE 4.4 Region upstream of putative uORF also contributes to VAR2CSA translation repression, suggesting that repressive element begins upstream of and extends into "uORF."

Comparison of PfIVT activity of firefly luciferase reporter constructs reveals that the 5'UTR segment prior to the putative "uORF" is equally important for inhibition of VAR2CSA translation to the "uORF." Together with ribosome profiling and the observation that the uORF is not translated, this suggests the existence of an alternate repressive overlapping the annotated uORF:
(A) wildtype W2 VAR2CSA 5'UTR
(B) W2 VAR2CSA 5'UTR with uORF deletion
(C) W2 VAR2CSA 5'UTR with deletion of region prior to uORF

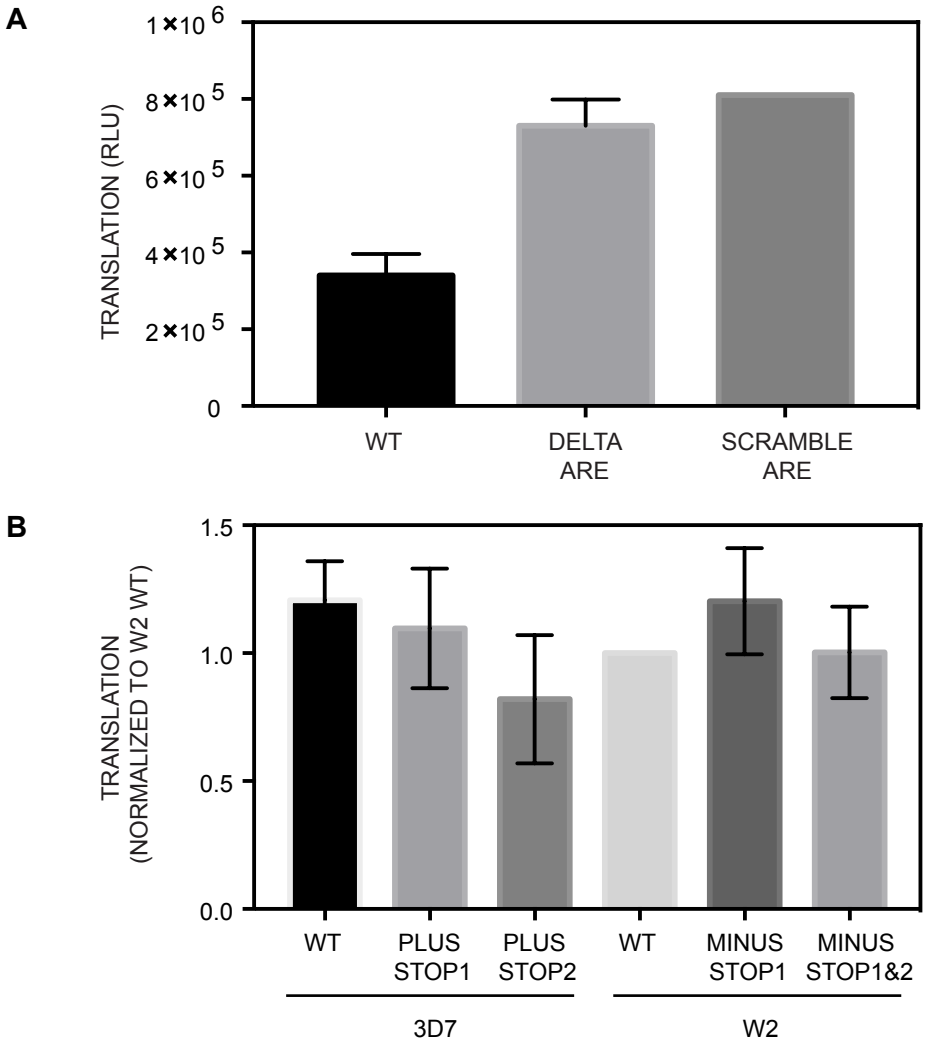

FIGURE 4.5 A novel element, the ARE (Alternative Repressive Element), contributes to repression of VAR2CSA translation in a sequence-dependent manner

(A) Comparison of PfIVT activity of nanoluciferase reporter constructs demonstrates sequence-specificity of ARE-dependent translational repression.
Constructs, from left to right: (1) wildtype W2 VAR2CSA 5'UTR, (2) W2 VAR2CSA 5'UTR with ARE deleted, (3) W2 VAR2CSA 5'UTR with scramble ARE
(B) Comparison of PfIVT activity of nanoluciferase reporter constructs suggests repressive role of additional STOP codons related to insertion of ARE. The left 3 constructs are the wildtype and mutant 3D7 VAR2CSA 5'UTR, the right 3 constructs are the wildtype and mutant W2 VAR2CSA 5'UTR. Translation is normalized to wildtype W2.

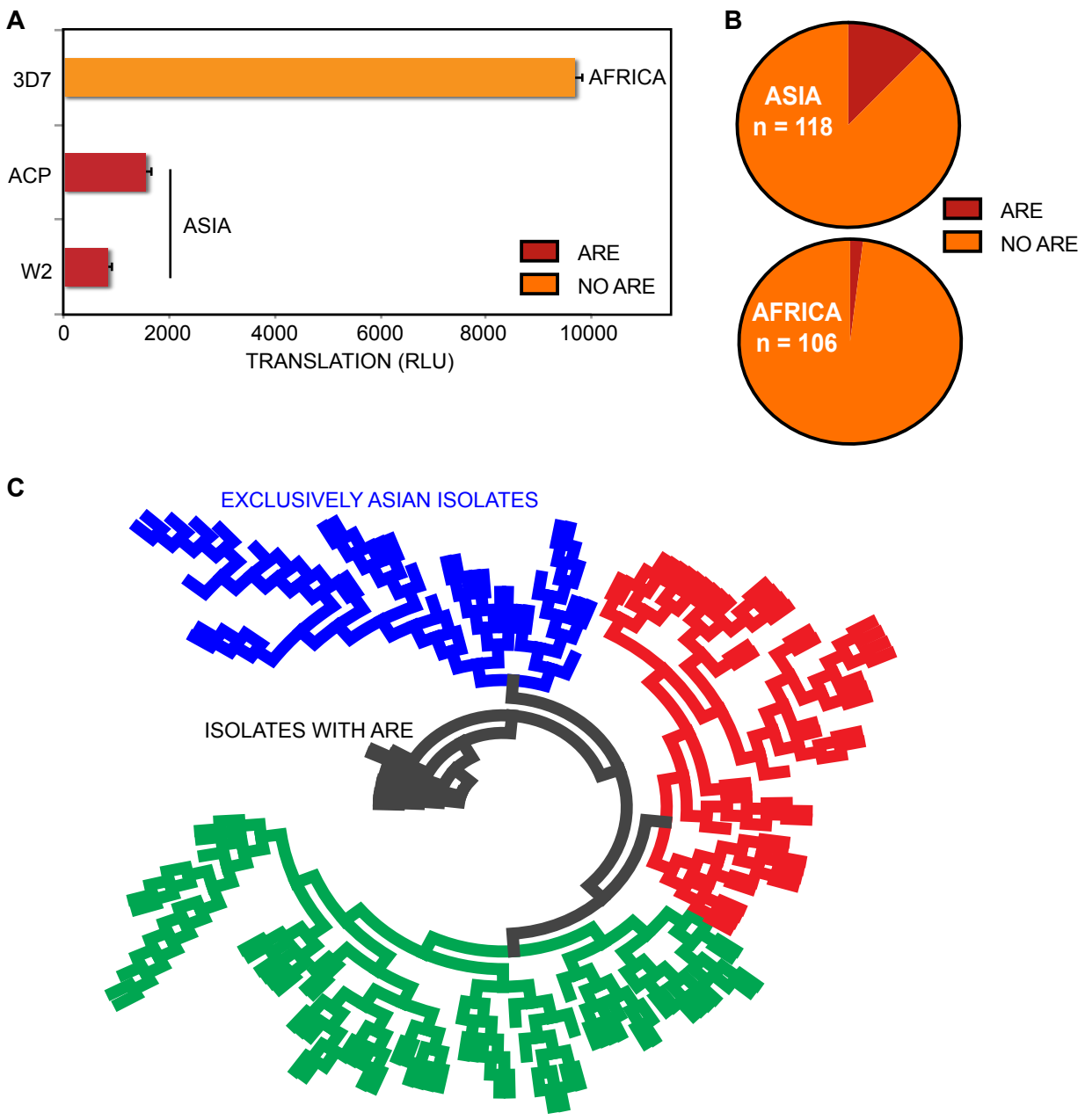

FIGURE 4.6 Geographic selection of the ARE.

(A) PfIVT activity of firefly luciferase reporter constructs with wildtype VAR2CSA 5'UTR from 3 different laboratory strains: 3D7 (African origin), ACPGFP (Asian origin), and W2 (Asian origin). Red bars: presence of ARE. Orange bars: no ARE. **(B)** Pie charts showing relative prevalence of ARE in 224 primary patient sample sequences. 12 of 118 Asian samples contain the ARE, compared to 2 of 106 African samples. Red: ARE. Orange: no ARE. **(C)** Phylogenetic tree constructed from alignment of VAR2CSA 5'UTRs from 224 primary patient sample sequences.

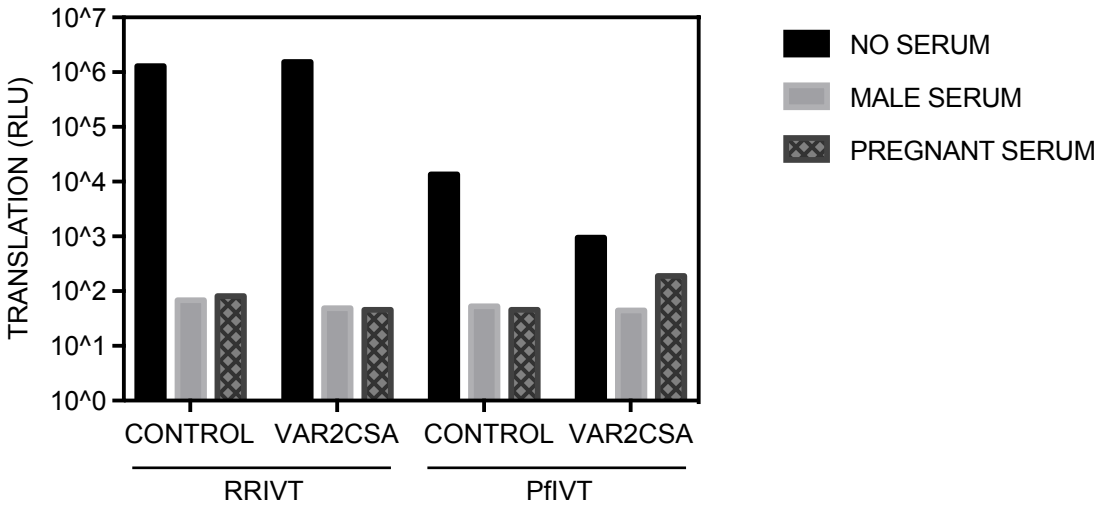

FIGURE 4.7 Maternal serum from 2nd trimester pregnancy specifically induces translation of VAR2CSA in *P. falciparum* but not rabbit reticulocyte *in vitro* assays.

Effect of serum addition to *in vitro* translation assays, comparing control (EBA-175 5'UTR) W2 VAR2CSA wildtype 5'UTR firefly luciferase reporter constructs. Water (no serum), male control serum, or maternal serum from 2nd trimester donor added to reactions. Assay performed in PfIVT and commercially available rabbit reticulocyte (RRIVT) systems.

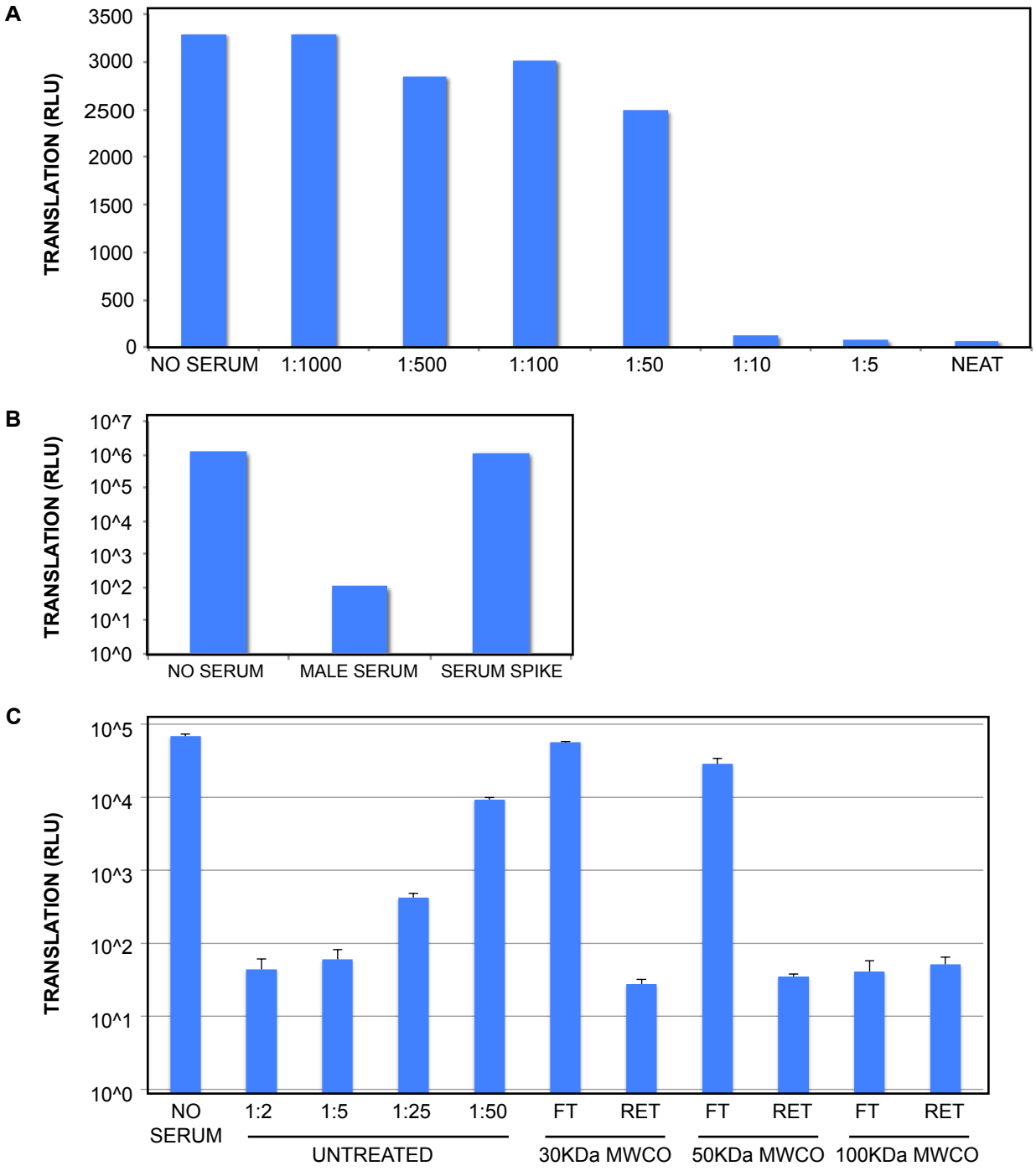

FIGURE 4.8 General inhibition of in vitro translation assay by serum can be removed through molecular weight cutoff filtration.

(A) Dilution series of male serum in PfIVT assay demonstrates general inhibition of assay by serum. **(B)** Addition of male serum to PfIVT assay at beginning of 120min incubation inhibits translation, while a spike-in of male serum to a parallel reaction at 105minutes of a 120min incubation has no effect, indicating that serum inhibits the PfIVT reaction itself and not luciferase activity. **(C)** Effect of flow-through and retentate of serum filtered through different molecular weight cutoffs. All assays performed using EBA175 5'UTR control reporter.

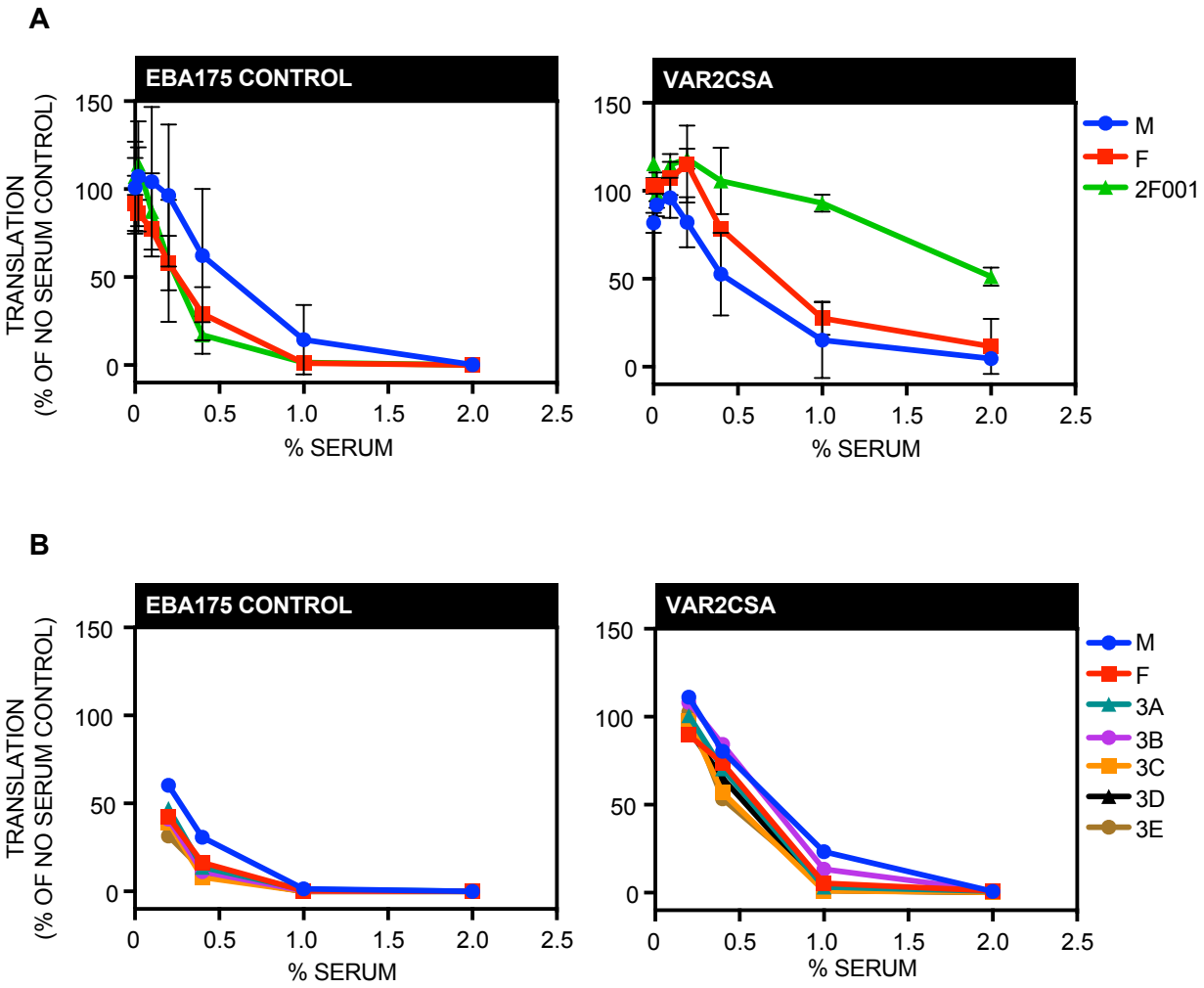

FIGURE 4.9 Maternal serum from 3rd trimester of pregnancy does not induce VAR2CSA translation.

(A) Titration of male (M, blue circles), nonpregnant female (F, red squares), and 2nd trimester pregnant female (2F001, green triangles) sera in the PfIVT assay with either control (EBA-175 5'UTR) reporter construct (left panel) or W2 WT VAR2CSA 5'UTR reporter. (B) Titration of male (M, blue circles), nonpregnant female (F, red squares), and 3rd trimester pregnant female (3A-E) sera in the PfIVT assay with either control (EBA-175 5'UTR) reporter construct (left panel) or W2 WT VAR2CSA 5'UTR reporter. All values translation values normalized to no serum control reactions.

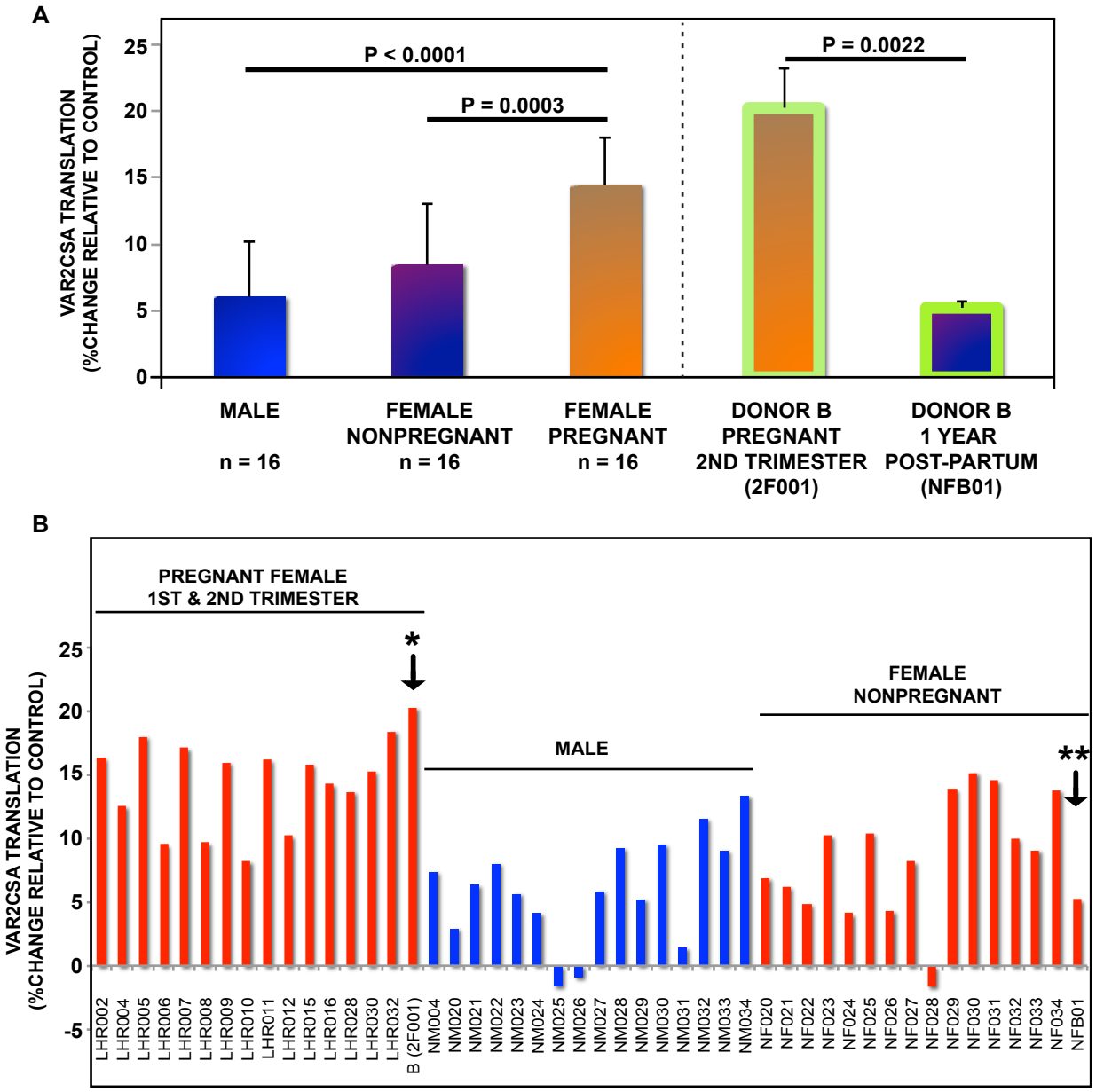

FIGURE 4.10 Maternal serum from 1st and 2nd trimesters of pregnancy induces translation of VAR2CSA.

Effect of male, nonpregnant female, and 1st/2nd trimester pregnancy serum on VAR2CSA translation in the PfIVT assay. n = 16 for all groups. All VAR2CSA translation values graphed as percent change relative to EBA-175 control. (A) Columns to left of dashed line: averaged values for each serum group. Columns to right of dashed line: serum from the same donor during the 2nd trimester of pregnancy (orange bar with green outline) and 1 year after delivering (blue bar with green outline). (B) Individual serum samples. arrows indicate serum samples from the same donor during the 2nd trimester of pregnancy (*) and 1 year after delivering (**)

TABLE 4.1 Mass Spectrometry Results for 2nd Trimester Pregnancy Serum

Top hits identified in 2nd trimester pregnancy serum sample but not male control.

Gene Name	Protein Name	Protein MW	Species	Number of Unique Peptides	Total Peptide Count	% Coverage	Best Discovery Score	Best Expected Value
CA1	Carbonic anhydrase 1	28870.4	HUMAN	4	5	15.3	6.68	8.90E-12
LDHA	L-lactate dehydrogenase A chain	36689.1	HUMAN	2	2	6.6	2.95	1.30E-07

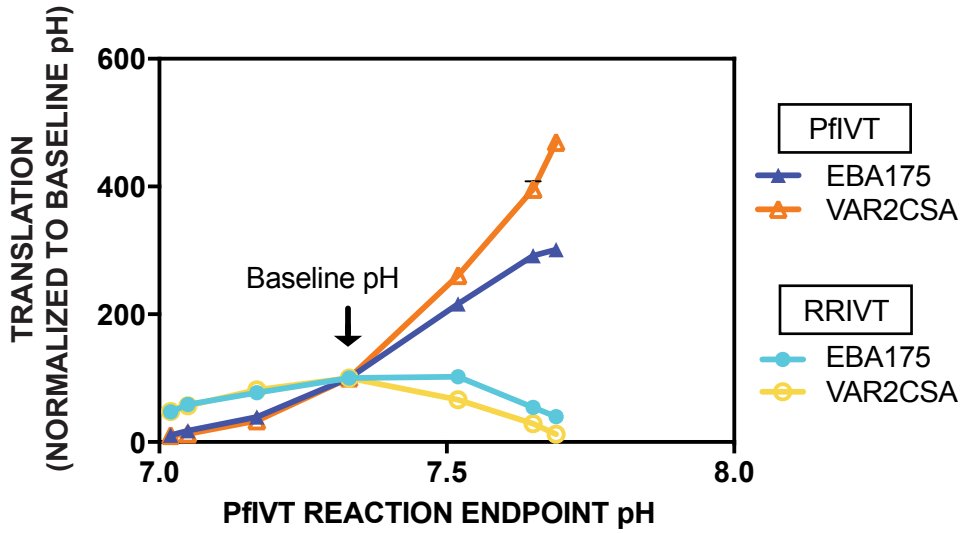

FIGURE 4.11 Increasing pH increases translation of VAR2CSA in PfIVT but not RRIVT

Translation of control (EBA-175 5'UTR; blue, closed symbols) or VAR2CSA 5'UTR (orange, open symbols) reporter construct in either P. falciparum in vitro translation assay (PfIVT; triangles) or rabbit reticulocyte in vitro translation assay (RRIVT; circles). pH of reactions determined at using microprobe at endpoint of reaction. Translation normalized to activity at baseline pH. Arrow indicates standard reaction condition (baseline pH).

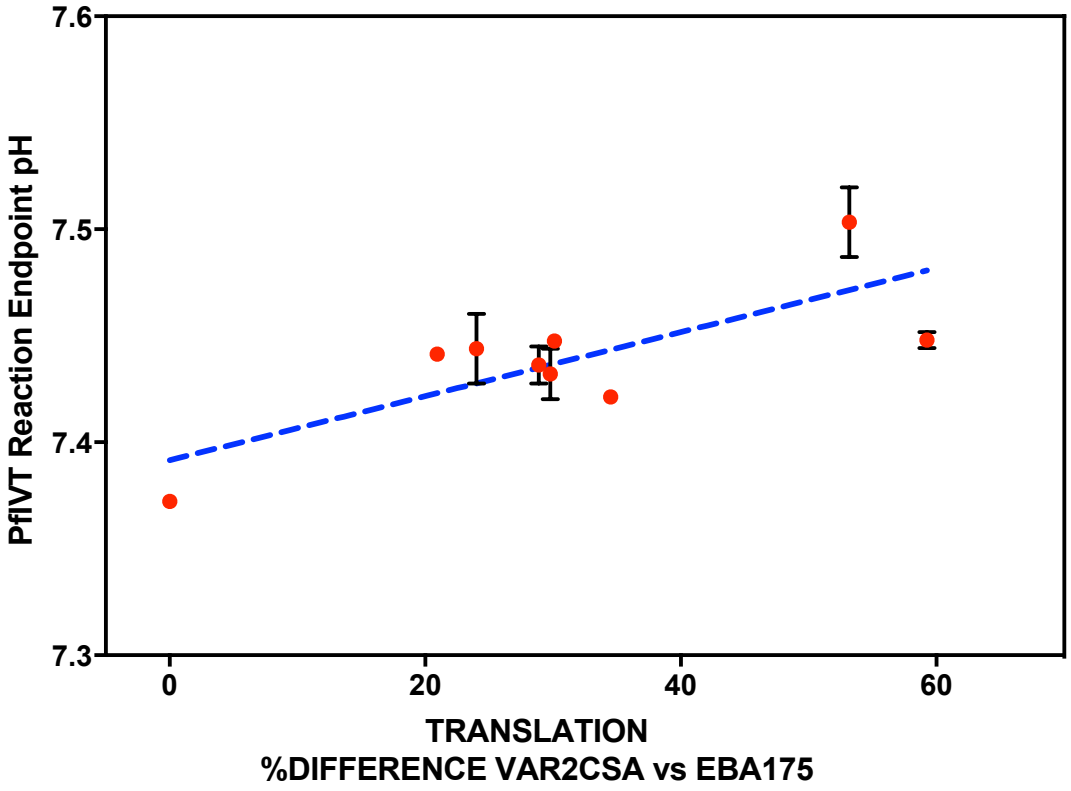

FIGURE 4.12 Increased translation of VAR2CSA with increasing pH of PfIVT reactions with added serum.

Translation of VAR2CSA in PfIVT reactions with serum samples added, plotted against pH measured at the reaction endpoint.

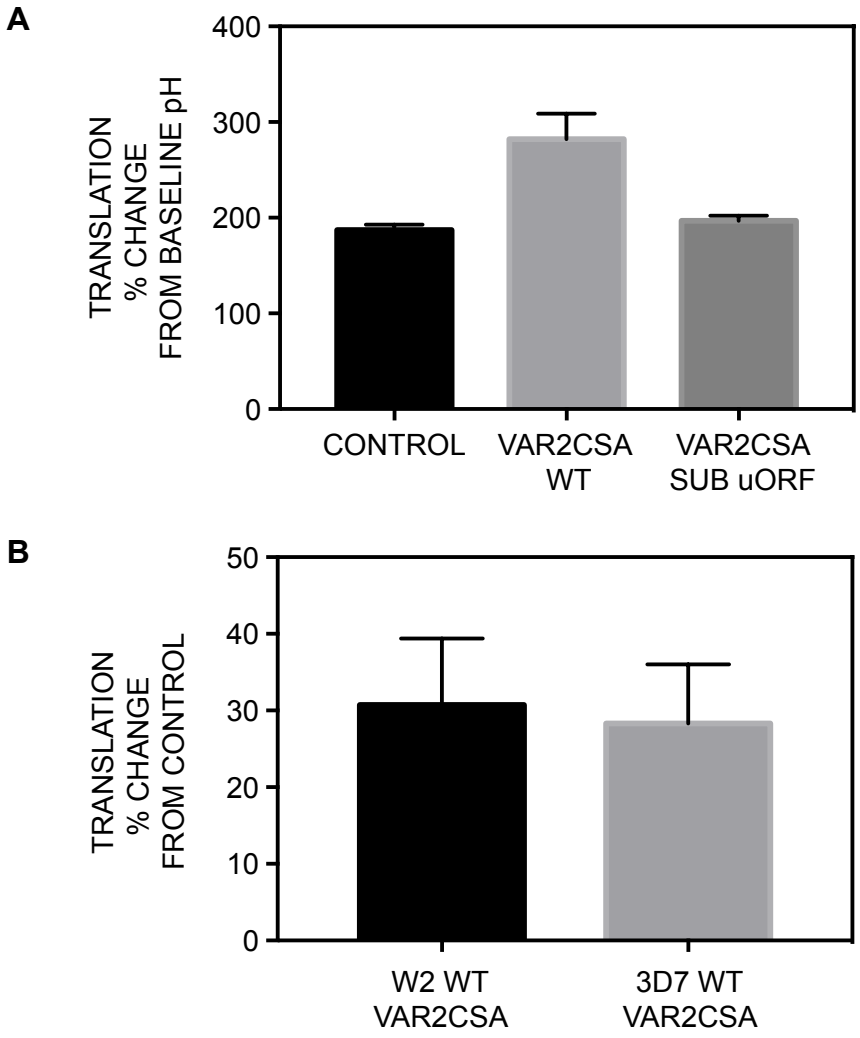

FIGURE 4.13 Putative "uORF" and ARE act independently; only uORF involved in pH/pregnancy response.

(A) Comparison of translation in PfIVT of Control (EBA-175 5'UTR), WT W2 VAR2CSA 5'UTR, and W2 VAR2CSA 5'UTR with a substituted uORF in response to high pH (pH7.69). Translation graphed as percent change from baseline pH reaction.
(B) Comparison of translation in PfIVT of W2 WT VAR2CSA 5'UTR and 3D7 WT VAR2CSA 5'UTR with addition of serum from 2nd trimester pregnancy (from Donor B). Translation graphed as percent change relative to control (EBA-175) with addition of serum.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

The development and careful optimization of the *P. falciparum in vitro* translation (PfIVT) assay has established the only direct measure of *Plasmodium* protein synthesis published to date. The PfIVT assay has proven a robust, reproducible, high throughput tool for screening inhibitors of *P. falciparum* translation and identifying potential drug candidates to combat malaria. Importantly, the method has also enabled the study of complex biology involving regulation of translation, such as that involved in placental malaria infection by *P. falciparum.*

5.1 PLASMODIUM FALCIPARUM GLOBAL TRANSLATION

Comprehensive characterization of the PfIVT assay with a broad panel of translation inhibitors, as well as control compounds targeting cellular processes other than inhibition, verified that the assay recapitulates *in vitro* the results that are observed in living parasites. The PfIVT assay specifically measures cytoplasmic *P. falciparum* ribosome activity, rather than that of mitochondrial or apicoplast ribosomes, and, moreover, specifically detects inhibitors of both translation initiation and elongation, as well as inhibitors of non-ribosomal components of the translational apparatus, while accurately differentiating such direct inhibitors of translation from the experimental artifacts that plague indirect methods of probing translation, such as S35-radiolabel incorporation assays. Having thoroughly verified the specificity of the PfIVT assay, we utilized it to investigate potential mechanism of action for the many clinically used

antimalarials for which the mechanisms are unknown, unclear, or in dispute. In so doing, we discovered that none of the antimalarials tested were direct inhibitors of *P. falciparum* translation. This is, in fact, a very encouraging result, as it means that parasites are unlikely to have developed resistance to translation inhibitors, since none of the currently approved drugs act on the translation machinery. Combined with the impressive pre-clinical results of the highly selective inhibitor of *P. falciparum* translation, DDD107498, this highlights the promising potential of targeting the parasite translational apparatus for newer generations of antimalarial drugs.

Importantly, the results of our screen of existing antimalarials also demonstrated, quite clearly, that mefloquine does not directly inhibit the *P. falciparum* ribosome, nor the extra-ribosomal components of the parasite translational apparatus. This result conflicts with a recent report that mefloquine binds the *P. falciparum* 80S ribosome in an antagonistic manner, however the authors' conclusions were based on cryo-EM performed under non-physiologic conditions and the indirect measure of S35 incorporation. Our findings highlight the importance of applying direct, functional measures when confirming the specific target and mechanism of action of a drug. Malaria remains a deadly disease and a significant global health problem; particularly in the current setting of decreasing funding for research and prevention of the disease, it is important that resources are not spent following an incorrect path. An obvious future direction is to continue work to determine the actual target of mefloquine. Follow-up studies to confirm and elucidate my preliminary calcium homeostasis data, as well as efforts to identify the true target of mefloquine through generation of drug-resistant mutants in combination with EMS mutagenesis or an inhibitor of the transporter protein

PfMDR1 (thereby hopefully circumventing the development of *Pfmdr1* copy number amplification most often associated with resistance to mefloquine), are promising avenues of study for determining the true mechanism of action of mefloquine, with the goal of informing future drug development efforts.

5.2 PLASMODIUM FALCIPARUM TRANSLATIONAL REGULATION OF VAR2CSA

Because the *P. falciparum* parasite exhibits a unique biology specifically when in a pregnant host, namely, the expression of the protein VAR2CSA, which contributes to establishment of a placental infection by the parasite, this presents a potential beneficial therapeutic window. In theory, if one could target and inhibit the expression of VAR2CSA, it would be possible to prevent placental infections, and potentially improve pregnancy outcomes, even in settings in which parasites are resistant to existing antimalarial therapies. My investigation into the translational regulation of VAR2CSA has identified both cis- and trans-acting factors contributing to the repression of VAR2CSA translation in a nonpregnant host, and as well as its induction in a pregnant host. However, additional study is required to further elucidate the mechanisms of regulation, as well as identify the full cohort of factors involved in this regulation, with the hopes of identifying potential therapeutic targets.

Utilizing serum samples from pregnant women, as well as from male and nonpregnant female controls, I determined that increased pH at the maternal surface of the placenta may constitute the “on” switch for translation; in essence, it is this more basic translation that the parasite senses, how it “knows” that its host is pregnant and

should therefore begin translation of VAR2CSA. However, more data is required to confirm this finding. Collecting samples from pregnant donors proved to be very challenging, and performing PfIVT studies with primary patient samples was even more demanding; the assay can be very finicky with addition of serum, and sample quality and condition was a limiting factor in many instances. Analysis of a newer, larger panel of serum samples, better controlled for proper storage to limit degradation, will be very important. Some of the variation seen within the pregnant sera group may be due to a gestational age effect (women are more susceptible to malaria earlier in pregnancy), and there may also be a contribution of gravidity (women are also more likely to develop malaria in their first than in subsequent pregnancies)(2,3,5). This newer set of serum should include samples from pregnant donors across the full span of gestation, divided into 6 groups for simplicity ("early" and "late" for each trimester): weeks 2-6, weeks 7-12, weeks 13-19, weeks 20-26, weeks 27-33, and weeks 34-40. Additionally, for each gestational group, there should be a subset of samples from primigravid women (women experiencing their first pregnancy) and a subset of samples from multigravida women (women who have been pregnant at least once before). Similarly, variation within the nonpregnant female control group may have been resulted from hormonal birth control status of the donors (it is possible that the hormones mimic an aspect of pregnancy to which the parasite responds, or influence the biology of the parasite in some other way); the newer set of controls should contain a broad panel of samples from donors who are taking hormonal birth control as well as a panel of samples from donors who are not taking hormonal birth control. Most promisingly, maternal serum samples from women with confirmed placental malaria cases should be tested in the

PfIVT assay, as it stands to reason that such samples would provide the best chance of identifying the factor(s) that lead to induction of VAR2SCA translation.

In addition to determining the effect of hormonal birth control, gestational age, and gravidity on induction of parasite translation, further study of the apparent pH effect and the candidate enzymes contributing to the increase in pH is warranted. pH measurements thus far have been performed only at the endpoint of the PfIVT reaction, just prior to measuring luciferase activity, and only a small subset of PfIVT reactions with serum were assessed for pH. Thus, pH measurements of naïve serum, as well as at the beginning (or an early time point) of the PfIVT reaction may be informative. In elucidating the effect of pH itself, it would be useful to further increase the pH of the reaction, as it does not appear that the pH-dependent increase in VAR2CSA translation has reached saturation in the pH range tested; an alternative buffer to HEPES will likely be required for these assays. Importantly, further study to determine whether LDHA and/or CA1 are truly the factors responsible for the increase in pH and induction of VAR2CSA translation is necessary. While I conducted preliminary PfIVT experiments with addition of both LDHA and CA1, with neither showing an effect, it is likely that the experimental conditions require optimization, and that the CA1 assays, in particular, need to be conducted in a closed system to limit the effect of CO_2 gas exchange during incubation. Additionally, once more “positive” pregnant serum samples (those inducing VAR2CSA translation) are identified, mass spectrometry analysis should be conducted on these samples, to see if the “hits’ of LDHA and CA1 are confirmed, or if other candidate proteins are identified. Importantly, these positive pregnant sera tested (pooled, if necessary) in the PfIVT assay with addition of inhibitors of LDHA and CA1, to

determine if inhibition of either enzyme in the serum samples negates the effect of the pregnant serum on VAR2CSA translation. Also, given the challenges associated with obtaining serum samples from pregnant donors, it is worth revisiting utilizing conditioned media from primary placental cultures as a substitute for serum – my previous efforts were inconclusive and hampered by inhibitory effects of the conditioned media in the PfIVT assay, but it is possible that this can be optimized and significantly improved, particularly with the lessons that have been learned along the way.

With regards to cis-acting regulatory factors, I identified two sequence elements within the 5'UTR of VAR2CSA that appear to contribute to repression of VAR2CSA translation via two distinct mechanisms: an element encompassing the putative "uORF" (or a segment of the 5' end of the putative "uORF") and a novel element downstream of the putative "uORF," which I have denoted the ARE. Many questions remain regarding the activity of these two elements, requiring a more thorough inquiry of the elements of the 5'UTR. When I embarked on this project, we were uncertain of the true transcription start site (TSS) of VAR2CSA – we now know that the TSS is farther upstream than we had determined, and so this additional 5' segment needs to be added to all of the existing VAR2CSA reporter constructs (97). In addition, several of the more recent assays have been performed utilizing a nanoluciferase reporter, which we now understand to be too robust for these assays comparing translation efficiencies of different 5'UTRs; the linear range yields a very small kinetic window, decreasing the potential fold changes observed between constructs, and making data difficult to interpret. An ongoing focus is to exchange firefly luciferase for the reporter in all current nanoluciferase constructs, and repeat the assays (such as the assay investigating the

role of start and stop codons surrounding the ARE). Further dissection of the 5'UTR, particularly determining the role (if any) of the various start codons upstream of the "uORF," determining the bounds of the element encompassing the "uORF," and determining the function of this element, since the putative "uORF" is not acting as canonical uORF (it is not inhibiting translation downstream by being translated itself), as well as further study of the function of the ARE (investigating start and stop codons, inverting the ARE, tiling in multiple AREs to determine whether ARE-dependent inhibition can be increased, etc.) are all in order. Also important will be determining whether the location of these repressive elements, either with respect to each other, or to the ORF itself affects their function. In addition, I have designed alternate scramble uORFs, optimizing for different criteria (i.e. maintaining or ablating start and stop codons within, scrambling nucleotide sequence vs. scrambling amino acid sequence, etc.). Testing this larger library of VAR2CSA 5'UTR mutant reporter constructs in the PfIVT under various pH and serum conditions will yield vital information as to the regulatory mechanism of the VAR2CSA 5'UTR. In addition, small-scale ribosome profiling of PfIVT reactions under various conditions – different pH, with and without pregnancy serum – comparing the different reporter constructs would provide information as to how the different sequence elements are contributing to ribosomal occupancy, and therefore translation, in response to changes associated with pregnancy.

One especially interesting finding of this study was the identification of the ARE, and, in particular, its apparent geographic distribution, with the ARE being much more prevalent in Asian than in African samples, inversely correlating with geographic prevalence of placental malaria. In addition to the assays investigating ARE function

noted above, it would be useful to perform ribosome profiling from cultures of the 3D7 strain, as well as its parent strain, NF54, to compare ribosome profiles across the VAR2CSA transcript to those seen in W2, and determine how the presence or absence of the ARE affects ribosomal occupancy in living parasites. Somewhat surprisingly, the VAR2CSA 5'UTR from the NF54 line seems to yield very different translational activity from the 3D7 VAR2CSA 5'UTR in the PfIVT assay. This result needs to be confirmed, but if it does repeat, differences between the two 5'UTRs will need to be probed to determine what is necessary and sufficient to confer repression of translation, as there are very few sequence differences between the two. Another interesting study to undertake would be analysis of the VAR2CSA 5'UTRs from patients with confirmed placental malaria, and comparison with those from non-placental malaria cases (i.e. in men and children) from the same period and area of transmission; if the presence of the ARE *in vivo* presents a barrier to expression of VAR2CSA, then one would anticipate that the ARE would be found at lower rates in parasites from women with placental malaria than in the rest of the infected population. In addition, efforts are currently being undertaken to determine whether the 2 ARE-containing patient samples collected in Africa are truly of African origin.

Importantly, it is likely that there is at least one binding partner involved in the repression and subsequent de-repression of VAR2CSA translation. Given the *P. falciparum* specificity of both the repression of VAR2CSA translation, and the increase in VAR2CSA translation with increasing pH, it is very unlikely that these effects are due solely to secondary structure of the 5'UTR; rather, there is likely an interacting protein, or perhaps even another RNA moiety, involved. Of additional interest is the apparent

periodicity of ribosomal peaks (major and minor) across the element encompassing the "uORF." The periodicity is reminiscent of "wrapping" or folding of the 5'UTR by a binding partner, leaving only certain portions exposed, similar to chromatin bundling by histones. An important next step in the study of VAR2CSA translational regulation will be mRNA pull-down of the various VAR2CSA reporter constructs from the PfIVT assay, at varying pH, and with and without pregnant serum, with the hopes of identifying the relevant binding partner(s) and the conditions under which binding is altered.

Another interesting future direction would be to consider the evolution of the regulatory elements of the VAR2CSA 5'UTR, and the selective pressures that might drive said evolution. Interestingly, the VAR2CSA 5'UTR is highly conserved, and much more conserved than the VAR2CSA ORF, which is highly polymorphic, suggesting the importance of the VAR2CSA 5'UTR in regulation of the gene (89,90). Similarly, the sequence of the ARE is highly conserved in the parasites that contain it. The apparent geographic structure of VAR2CSA 5'UTRs from patient samples when building a phylogenetic tree suggests points to unique geographic selection or drift, perhaps due to different rates or periodicity of malaria transmission in different regions. It is interesting to think of the balance between evolutionary pressures that might affect expression of VAR2CSA: after all, there is a great benefit to the parasite to be able to quickly "capitalize" on finding itself in a pregnant host and exploit the placental niche, on the other hand, the risk of too easily expressing VAR2CSA outside of the setting of pregnancy would expose the parasite to immune system. One might hypothesize that the ARE was initially present in all parasites, but was lost over time in conditions in which it was more beneficial to the parasite to be able to express as much VAR2CSA as

possible, as quickly as possible. Additionally, in a recent report, VAR2CSA-specific antibodies have been identified in men and children in Colombia (98). This finding must be taken with a grain of salt, as many studies of antibodies against the various VAR proteins, including VAR2CSA, are found to be flawed due to cross-reactivity of antibodies owing to great similarity in the various VAR proteins. However, should the finding hold true, it begs the question, is the VAR2CSA 5'UTR in this region different than those that we have studied? Are there regulatory elements that are missing? Analyzing samples from these patients could be very informative as to the regulatory biology of VAR2CSA expression.

The most important question to ask among all of these is "cool, but does this really happen in a living parasite?" Determining what factors induce expression of VAR2CSA in culture would be most convincing as to what is occurring biologically in patients. I have spent some time culturing parasites at increasingly high pH, and then attempting to measure VAR2CSA expression via panning or flow cytometry. However, these efforts have been limited by the lack of specific anti-VAR2CSA antibodies, the technical challenges of panning, and related lack of appropriate controls. One important finding resulting from these efforts, however, is that the parasites can survive and reinvade for many cycles at much higher culture pH than we anticipated (as high as pH9 media with twice daily feedings), and that, despite extremely efficient buffering by both the erythrocytes and parasites, it does appear that pH could be raised enough in the parasite to achieve VAR2CSA-specific induction of translation, but this will require further study. Most importantly, efforts are currently underway to utilize CRISPR/Cas9 to introduce a fluorescent reporter into our parasites, so VAR2CSA expression can be

monitored in culture. This will allow assaying of different pH conditions of culture; addition of LDHA, CA1, and any other candidate factors to culture; and even co-culture with placental tissues.

5.3 CONCLUDING REMARKS

In summary, my thesis work yielded many interesting findings and observations, regarding both “normal” global translation and translational regulation of a specific gene under specific conditions, that warrant further study. The optimization and careful characterization of the PfIVT assay resulted in a robust, highly reproducible method with a myriad of potential uses in the study of *P. falciparum* translation. In particular, the definitive determination that mefloquine does not directly inhibit the *P. falciparum* 80S ribosome is especially important, as it may directly inform future drug development efforts and related research. Follow up on my preliminary data regarding identification of actual target of mefloquine would also be of great use. Additionally, further characterization and elucidation of the cis- and trans-acting factors involved in regulation of VAR2CSA in placental malaria will be very informative. It is my hope that this work may one day have implications for not only a better understanding of parasite biology in the setting of pregnancy, but for developing targeted therapies to treat the parasite, or at least mitigate its negative effects in pregnancy.

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