Role of STEVOR and its interacting partners in parasite virulence

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Role of STEVOR and its interacting partners in parasite virulence

Radosław Igor Omelianczyk

SCHOOL OF BIOLOGICAL SCIENCES

2019

Role of STEVOR and its interacting partners in parasite virulence

Radosław Igor Omelianczyk

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2019

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Abstract

The genomes of *Plasmodium* spp. encode a number of different multigene families that are thought to play a critical role for survival. However, with the exception of the *P. falciparum* var genes, very little is known about the biological roles of any of the other multigene families. The *stevor* family includes about 40 genes and is therefore the smallest family of variant surface antigens in *P. falciparum*. While some of its members have been shown to interact with glycophorin C, some others are presented at the apical tip of merozoites where they are involved in invasion. However, much of the diversity within the family is still understudied.

The first part of this work describes our efforts to identify STEVOR with novel binding properties to red blood cells. To this end, amino acid sequence alignment provided information about the diversity within the family. In order to cover the entire diversity within the *stevor* multigene family, a large subset of different STEVOR were expressed on the surface of mammalian cells or recombinantly in bacteria. We successfully identified one *stevor* with atypical binding behaviour to red blood cells, providing us with a lead candidate for further studies.

The second part of this work describes efforts to develop a platform to study members of multigene families in *P. falciparum*. Using the recently developed Selection Linked Integration method, we have been able to activate the expression of a single member of a multigene family of our choice from its endogenous promoter. We characterize parasites expressing specific members of the *var*, *rifin* and *stevor* multigene families in terms of protein localization, function and epigenetic control mechanisms underlying their expression. We show that endogenous tagging of a PfEMP1 does not disrupt transport of the protein to the surface of the host red blood cell, suggesting that the protein transport is not altered by the knock-in method. Furthermore, knock-in of two previously described RIFIN causes the parasites to become resistant to natural killer cell killing, proving functionality of the proteins. Finally, knock-in of three different STEVOR highlights the diversity observed in this multigene family. Each of the proteins shows a different subcellular localization, with one being exposed in the surface of the

infected red blood cell, one remaining within the Maurer's Clefts and one being expressed as a truncation.

Using microarray analysis, we gain new insights into the regulatory mechanisms within and between multigene families. We can show that knock-in of *stevor* causes monoallelic expression, suggesting mutual exclusive control within this family. In contrast, *rifin* do not appear to have any level of control mechanisms. Furthermore, we observe cross-talk between multigene families as expression from a genomic locus causes upregulation of some, but not all, adjacent genes. We show that similar observations have been reported previously, suggesting true interaction instead of artefacts introduced by the knock-in

Finally, we studied intracellular interaction partners of STEVOR utilizing two independent approaches: co-immunoprecipitation and proximity labelling by BioID. We show that both the surface exposed and the Maurer's Cleft resident STEVOR interact with unique subsets of proteins. While the surface exposed protein appears to be incorporated into the new permeability complex together with other small variant antigens, the other one is associated with lipid rafts within the Clefts.

1. Introduction

1.1. Evolution and history and *Plasmodium*

The first historical mention of malaria dates as far back as 2700 BC. The Chinese Nei Ching (Canon of Medicine) mentions paroxysmal fevers associated with enlarged spleens and a tendency toward epidemic occurrence (Hoeppli, 1959). Further mentions of the disease can be found in India. Vedic (1500 BC to 800 BC) and Brahmanic (800 BC to 100 AD) scriptures both mention fevers which occurred in periodic intervals, either daily or on alternating days (Bruce-Chwatt, 1965). Egyptian (Ebbell, 1937) and Sumerian (Sarton, 1919) texts dating back to 2000 BC to 1500 BC describe the same periodic fevers combined with splenomegaly. The first mention of symptoms attributed to malaria around the Mediterranean originates from Greece (500 BC) (Hippocrates, Jones, Whithington, & Heraclitus, 1957; Sarton, 1919) and Italy (200 BC) (Celli & Celli, 1933). The parasite itself originated most likely hundreds of thousands years earlier. Genetic evidence suggests that the common *Plasmodium* ancestor was once a free-living, chloroplast-containing protozoan (Wilson & Williamson, 1997). During evolution, the chloroplast morphed into the apicoplast, present in most members of the phylum of Apicomplexa, to which *Plasmodium* belongs. These ancestors are believed to have lived in the guts of aquatic insect larvae. These insects themselves evolved into flies which later on gave rise to the taxa of Dipterans, which today includes mosquitoes, while the parasite itself found ways to evade the mosquito immune response (Collins et al., 1986; Hurd, Taylor, Adams, Underhill, & Eggleston, 2005). Further co-evolution between the early parasite and its host led to the adaptation of the parasite which allowed it to survive in mammalian hosts, hence giving rise to the parasitic organism found today (Martinsen, Perkins, & Schall, 2008; Ricklefs, Fallon, & Bermingham, 2004).

Today, more than 25 different species of *Plasmodium* have been described and are known to infect primates (Garnham, 1966). Among those, 5 are known to infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi* and *P. ovale*. *P. falciparum* diverged from the other human infectious species up to 130 million years ago (Escalante & Ayala, 1995). Based on genetic evidence, it is more closely related to the chimpanzee-infecting *P. reichenowi*, and both of them are more related to the avian *P.*

gallinaceum than the other primate infecting species. Considering that Dipterans evolved around 150 million years ago, this separation happened at the very beginning of *Plasmodium* evolution. The separation of *P. reichenowi* and *P. falciparum* subsequently is thought to have happened some 4 to 10 million years ago (Escalante & Ayala, 1994; Escalante, Barrio, & Ayala, 1995; Escalante, Freeland, Collins, & Lal, 1998), coinciding with the branching of the human and the great ape line 5 million years ago (S. Kumar & Hedges, 1998).

In order for the parasite to sustain high rates of human instead of primate infection, the feeding pattern of the *Anopheles* mosquito is of crucial importance. While most zoophilic malaria vectors prefer to feed on other mammals as compared to humans (~80 – 90% of blood meals), malaria vectors in sub-Saharan Africa are outliers. Mosquitoes in these regions prefer humans to feed on and reach up to 100% feeding selection (Bruce-Chwatt, Garrett-Jones, & Weitz, 1966). This is an important observation considering that the chance of transmission is proportional to the square of blood meals taken on humans (Macdonald, 1957). One theory to explain this observation is linked to the agricultural revolution happening around the world some 5000 to 8000 years ago (Coluzzi, 1999; Otto et al., 2018). Human societies started to settle down and cultivate the land. This led not only to local accumulation of humans in settlements but also to agricultural related formation of stagnant water bodies which are breeding grounds for mosquito larvae. The difference between sub-Saharan and Asian settlements, which developed even earlier, is the lack of domesticated animals in Africa compared to the abundance found in Asia, hence driving the preference for human blood in sub-Saharan regions (Diamond, 1997).

Since human settlements apparently coerced the vector to mainly feed on humans, the parasite itself adapted to the new circumstance. Instead of infecting different monkey species, it was now restricted to only one mammalian host. *P. falciparum*, the species which today by far, has placed the heaviest burden on human society, was in the past one of the less competitive species. It used to go extinct in situations which still sustained other *Plasmodium* species (Mendis, Sina, Marchesini, & Carter, 2001). While the reasons are unclear, SNP analysis of modern-day *P. falciparum* populations suggests that the entire species went through a tight bottleneck some 5000 – 10000 years ago (Otto et al., 2018; Rich,

Licht, Hudson, & Ayala, 1998). Even though there are counter arguments against this theory, citing the high divergence in certain genes which is unlikely to have arisen in such a short timeframe (Hughes & Verra, 1998), the previously mentioned timeframe coincides with the selective pressure the parasites started to put on human populations which will be discussed further on.

By this point in history, human societies in sub-Saharan Africa were closely tied to the constant prevalence of *P. falciparum*. Since *P. falciparum* is, in contrast to the other human-infecting *Plasmodium* species, the cause of severe and commonly lethal outbreaks of the disease, it has been suggested that the parasite actively applies evolutionary pressure on human society (Cavalli-Sforza, Menozzi, & Piazza, 1994). Numerous human genetic diseases have been associated with protection against malarial infection, including Glucose-6-Phosphate dehydrogenase deficiency, sickle cell trait, ovalocytosis, thalassemias, mutations in Hemoglobin C and E and duffy negativity (Beet, 1946; Cattani, Gibson, Alpers, & Crane, 1987; Flint, Harding, Boyce, & Clegg, 1993; Garnham, 1966; Gilles et al., 1967; Modiano et al., 2001; Ray, Chatterjea, & Chaudhuri, 1964). Prevalence of these traits is tightly linked to regions where malaria is endemic. Even more so, with increasing malaria pressure, the occurrence of more detrimental genetic mutations such as ovalocytosis or sickle cell trait increases. This is most apparent for duffy negativity, which is driven mainly by the prevalence of *P. vivax* (Horuk et al., 1993). The receptor is essential for *P. vivax* merozoite invasion into red blood cells. In sub-Saharan Africa, the postulated ancestral breeding ground of *Plasmodium*, duffy negativity is conserved and affects 100% of the population, making the population immune to *P. vivax* (Miller, Mason, Clyde, & McGinniss, 1976). It is worth noting that the distribution of human genetic polymorphisms is different across the globe, with some regions being enriched in certain traits and not others (Flint et al., 1993; Tishkoff et al., 2001). This suggests that the spread of the disease happened after modern day human populations emerged from Africa and spread across the globe.

1.2. Epidemiology of *P. falciparum*

Today, people are at risk of contracting malaria in 92 countries (World Health Organization. Global Malaria Programme, 2018). Limiting factor for transmission is mainly temperature which has to allow for the propagation of the *Anopheles* mosquito as well as survival of the parasite within the insect host. 92% of the cases occur in sub-Saharan Africa, where 99.7% of the cases are caused by *P. falciparum*. 5% of the cases were reported from South-East Asia, 2% from the eastern Mediterranean region and 1% in the Americas. This amounts to 219 million cases in total reported in 2017. The numbers appear to stagnate after several years of decline (239 million in 2010 and 217 million in 2016). Similar to the number of reported cases, the number of malaria related deaths hit a plateau after a steady decline over the past 7 years (2010: 607000; 2016: 451000; 2017: 435000). The emergence of resistance to anti-malarial and anti-mosquito drugs is a possible explanation for this stagnation (Dondorp et al., 2009; World Health Organization., 2018). The unequal distribution of cases among the endemic countries places an especially high burden on only 5 countries which harbour almost half of all cases worldwide. Among those, Nigeria and the Democratic Republic of Congo together account for about 30% of all cases and deaths worldwide. Even though these numbers are lower than what they have been in the past, the diseases have created massive socio-economic costs which are difficult to estimate. This is especially due to the lethality of *P. falciparum* infection which results in endemic countries struggling with the implications of stable malaria transmission (Gallup & Sachs, 2001). In addition to direct deaths caused by the parasite itself, malaria infection increases the severity of other infections (Church & Maitland, 2014). Malaria eradication leads to decreases in mortality exceeding the predicted deaths directly caused by malaria, strengthening the argument for an interaction with other pathogens (Alles, Mendis, & Carter, 1998).

Countries where the diseases are endemic can be sub categorised into two groups: stable and unstable epidemic transmission. Stable transmission occurs if infectious bites are delivered at a rate which allows affected people to achieve full immunity to the disease. Generally, immunity to a particular strain that a person is infected with is acquired quickly (Jeffery, 1966; Wilson & Phillips, 1976). With

increased exposure, disease severity is reduced until absence of symptoms is achieved (Day & Marsh, 1991). Sterile immunity to *P. falciparum* on the other hand is achieved in only rare cases. Immunity against the disease as a whole is thought to arise by acquisition of immunity to a large diversity of different strains, thus explaining the necessity of multiple infections (Cadigan & Chaicumpa, 1969; Jeffery, 1966). In endemic regions, this usually occurs from the age of 10 onwards. This high level of immunity however requires regular (at least every few months) contact with the parasite (Mbogo et al., 1995). Without regular contact with the parasite, immunity quickly diminishes. Since immunity requires several inoculations against the parasite in order to drastically reduce disease outcome, and considering the lethality of *P. falciparum* infection, it is not surprising that most malaria related deaths (61%) occur in children below the age of 5 (World Health Organization. Global Malaria Programme, 2018). In regions with unstable malaria transmission, immunity to the disease will never fully develop. Since immunity takes several infections to evolve and regular contact with the parasite to persist, infrequent episodes of malaria cannot sustain full protective immunity. These circumstances are in fact much more dangerous for an individual since every infection is capable of causing severe disease (Trape, Peelman, & Morault-Peelman, 1985). To make matters even worse, the other human infectious *Plasmodium* species are capable of causing severe malaria in individuals without protection as well, potentially causing a drastic reduction in life expectancy (Dobson, 1994). Outbreaks of *P. falciparum* can therefore lead to massive epidemics with millions of cases and more than 100000 deaths within short time periods (Fontaine, Najjar, & Prince, 1961; Gramiccia & Hempel, 1972). Similar effects could be observed when people from non-malaria endemic regions travelled to areas with stable transmission (Watt, 1978).

1.3. Parasite life cycle and disease symptoms

As mentioned above, the limiting factor for malaria transmission is the ability of its host, the *Anopheles* mosquito, to thrive in the climate and to retain living parasites between two blood meals. During a blood meal of an infected mosquito, sporozoites are injected into the dermis. Even though the details of the following few hours are poorly understood, the parasite uses gliding motility to reach a blood vessel and to enter the blood stream. Once they reach the liver, they penetrate the outer layer, consisting of endothelial and Kupffer cells, and finally arrive and infect hepatocytes. It is noteworthy that even though the parasite passes through the barrier cells (Risco-Castillo et al., 2015), they only form a transient vacuole and leave the cell again instead of fully infecting it. In order to differentiate between cells that only require passing through and its target cell, the parasite senses several different surface receptors on hepatocytes which give the signal to infect the cell. In order to fully develop, the parasite engulfs itself in a parasitophorous vacuole, which is an additional membrane layer formed during invasion. After development within those cells for 7 days, up to 40000 merozoites per hepatocyte are released into the blood stream. These merozoites, once interacting with red blood cells, will initiate a process of several different steps which finally leads to an invasion of the red blood cell and enveloping of the parasite in its vacuole. Within the vacuole, the parasite will spend the next 48 hours maturing from ring through trophozoite to finally, the schizont stage. The asexual life cycle ends with the rupture of the infected red blood cell and release of 16-32 daughter merozoites. The periodic release of daughter cells every 48 hours, which is accompanied with some of the symptoms of the disease, is a hallmark of malaria which has been used to identify the illness in the ancient texts mentioned previously. Since asexual cells cannot survive in the mosquito, the parasite has to differentiate into specialised sexual gametes. A small subset of cells commits to sexual differentiation after sensing external stimuli such as high parasitemia, lack of nutrients and exposure to certain drugs. During gametocyte development, which takes 8-10 days, the parasite resides in the bone marrow and only re-enters the blood stream upon final maturation (Joice et al., 2014).

Figure 1: The life cycle of *Plasmodium falciparum* within the mammalian host.

During a blood meal, the female *Anopheles* mosquito injects sporozoites into the dermis of the mammalian host. These sporozoites travel to the liver where they invade hepatocytes. After approximately 10 days, merozoites are released which enter the bloodstream and start invading red blood cells. After invasion, the parasites mature from ring to trophozoite to schizont stage. During the latter two stages, the infected red blood cells shows both cytoadherence and rosetting. After 48 hours, the red blood cell bursts and releases between 16 and 32 daughter cells which will re-invade new red blood cells. A small subset of parasite will differentiate into sexual gametocytes. After maturation, which occurs in the bone marrow, gametocytes are released back into the blood stream where they can be taken up another mosquito. Adapted from (Wahlgren, Goel, & Akhouri, 2017)

The mild symptoms of the disease are almost always those of a generic febrile illness: headache, nausea, fatigue, muscle pain, sweating and rigors. Due to the 48-hour life cycle of the parasite, these symptoms often times occur in periodic intervals. If untreated, the spleen tends to swell up, resulting in the characteristic splenomegaly. Treatment with adequate antimalarials will lead to complete remission within only a few days. Considering that *P. falciparum* does not form any dormant forms, no

relapse should occur after a full medication cycle. Severe malaria includes dysfunction or failure of vital organs like lungs, liver or kidney, anaemia and “cerebral malaria” (Marsh et al., 1995). The latter one is often times caused by cytoadhesion of mature parasites in the microvascular endothelium in the brain (Taylor & Molyneux, 2015). Formation of rosettes, the binding of infected red blood cells to uninfected ones, causes further microvascular obstruction (Kaul, Roth, Nagel, Howard, & Handunnetti, 1991). Severe symptoms are generally caused by either obstruction of blood vessels due to sequestration, metabolic acidosis, over-induction of host immune responses and loss of blood cells due to parasite propagation (parasitemia can increase more than 10-fold every 48 hours) (Wassmer et al., 2015). The host body attempts to control parasitemia by activation of immune pathways that are designed to fight infections (Cunnington, Walther, & Riley, 2013). Considering that these pathways do not necessarily help with fighting malaria, an excessive immune response has been associated with severe malaria episodes (Kwiatkowski et al., 1990). Adding onto the prevalence of inflammatory cytokines released by immune cells, endothelial cells begin to secrete nitric oxide and angiopoietin 2- both are described markers of severe disease outcome (Hanson et al., 2015; Yeo et al., 2014). It has been described that interaction between parasite proteins and endothelial protein C receptor (EPCR) during sequestration can be a cause for the secretion (Turner et al., 2013). Sequestration describes the binding of infected red blood cells to endothelial cells in microvasculature. This is mediated by a vast array of parasite proteins which are displayed on the surface of the red blood cell. In addition to endothelial cells, binding to red blood cells and activated platelets has been described as well. Taken together, parasite mediated clumps of cells can cause obstruction of blood flow and inhibit oxygen supply to organs as well as metabolic acidosis and are therefore direct causes of severe disease symptoms (Dondorp et al., 2008).

1.4. The *Plasmodium falciparum* multigene families

One hallmark of *P. falciparum* is its repertoire of different multigene families. Even though genes within each family share high sequence homology, the parasite only expresses a small subset of them at any

given point in time. Among these families, 3 are commonly grouped as variant surface antigens (VSA): *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Howard et al., 1988), subtelomeric variant open reading frame (STEVOR) and repetitive interspersed repeats (RIFIN) (Cheng et al., 1998), encoded by the *var*, *stevor* and *rif* genes respectively. Given their distinctly smaller sizes, the latter two are termed 'small variant surface antigens' (sVSA). The genes for all three families are located in the subtelomeric regions of all 14 chromosomes (Gardner et al., 2002). Since the telomeres of *P. falciparum* do not encode for any essential genes, they have been hotspots for the generation of variability within each multigene family. During asexual mitosis (Claessens et al., 2014) and sexual fusion (Scherf, Lopez-Rubio, & Riviere, 2008), the genomic loci undergo duplications, translocations, mutations and deletions (Hamilton et al., 2017; Miles et al., 2016; Mok, Ribacke, Sherwood, & Wahlgren, 2008; Ribacke et al., 2007). These VSAs not only interact with host molecules on the surface of both RBCs as well as endothelial cells, they are predicted to help evade the host immune system through the generation of antigenic variation (Biggs et al., 1991; Reeder & Brown, 1996). Similar to *trypanosomes* (Borst et al., 1997) and *borrelia* (Stoenner, Dodd, & Larsen, 1982), the parasite only expresses a single member or a small subset of the family at a given time (Dzikowski, Frank, & Deitsch, 2006; Lavazec, Sanyal, & Templeton, 2007; Petter, Bonow, & Klinkert, 2008; Scherf et al., 1998; Smith et al., 1995). Switching to other members of the family changes the antigens displayed on the infected red blood cell surface, thus evading recognition by the humoral immune response. Not only does removal of PfEMP1 result in almost complete loss of antibody recognition, it has been shown that expression of all VSAs is downregulated in cultured parasites and in a splenectomised patient, highlighting the involvement of the immune system (Bachmann et al., 2009a; Chan et al., 2012). It would also explain the oscillating waves of parasitemia seen in chronic infections (Miller, Good, & Milon, 1994). Furthermore, sequestration removes late stage parasites from open blood circulation and therefore, avoids a splenic clearance.

1.5. PfEMP1

PfEMP1 is the most intensively studied multigene family, with about 60 genes in the 3D7 reference genome (Gardner et al., 2002). It has the highest variety of interacting partners, including endothelial and red blood cell receptors such as CD36, ICAM1, CSA, CR1, EPCR, heparan sulfate and thrombospondin (Baruch, Gormley, Ma, Howard, & Pasloske, 1996; Fried & Duffy, 1996; Howell et al., 2008; Robinson, Welch, & Smith, 2003; Turner et al., 2013). PfEMP1 is, so far, the only variant surface antigen identified which causes sequestration. The significance of sequestration for disease path ology explains the research focus on this multigene family. Furthermore, presence of PfEMP1 on infected red blood cells suppresses release of interferon gamma from peripheral blood mononuclear cells during early infection (D'Ombra et al., 2007). Early reports that PfEMP1 inhibits maturation of dendritic cells have been later questioned since this effect appears to be a dose dependant consequence of infected red blood cell presence (Elliott et al., 2007; Urban et al., 1999). PfEMP1s are large multidomain proteins with a molecular weight between 250kDa and 350kDa. Each gene includes two exons: exon 1 encodes for the variable part of the protein. This part is ultimately exposed on the surface of the infected red blood cell and is involved in antigenic variation as well as receptor interaction. Exon 2 on the other hand, encodes the highly conserved cytoplasmic acid terminal segment (ATS) domain (Baruch et al., 1997; Su et al., 1995). The intron itself includes a second, bi-directional promoter, which gives rise to long non-coding RNA (Epp, Li, Howitt, Chookajorn, & Deitsch, 2009). While PfEMP1s are usually anchored to knobs (membrane protrusions on the surface of infected red blood cells), knobs are not essential for PfEMP1 surface display (Horrocks et al., 2005; Waller, Nunomura, Cooke, Mohandas, & Coppel, 2002). Anchorage of some PfEMP1 (but interestingly not all) appears to involve LyMP, a PHIST protein which interacts both with the host cell cytoskeleton as well as the ATS domain (Oberli et al., 2016). Furthermore, correct surface display of PfEMP1 seems to be enhanced by a currently unknown serum lipoprotein (Frankland et al., 2007; Ribacke et al., 2013). In striking contrast, display of RIFINs and STEVOR is increased in parasites cultured in AlbuMAX - the serum substitute commonly used in *P. falciparum* culture (Tilly et al., 2015).

Phylogenetically, *var* genes have been grouped into 3 classes: *upsA*, *upsB* and *upsC*, depending on their upstream untranslated regions (Gardner et al., 2002). *Var* genes of the same group are commonly clustered at the same genomic locus. Most *var* genes are unique to a specific strain. Considering that most of the parasite antigens presented on the surface of infected red blood cells are part of a multigene family, and these proteins are different between strains, it is of little surprise that immunity to *P. falciparum* requires several infections to fully develop. There are however some *var* genes which are strain- transcendent. The most notable one among them is *var2csa* which causes sequestration in the placenta of pregnant women. Others are the pseudogene *var1csa* (the only *upsD* var).

Among the multigene families, expression control is the tightest for *var* genes. Each parasite expresses exactly one gene at a time (Chen et al., 1998; Scherf et al., 1998). Parasites can switch expression to a different *var* gene with switch rates of up to 2% *in vitro* but with no clear pattern (Frank, Dzikowski, Amulic, & Deitsch, 2007; Noble et al., 2013; Recker et al., 2011). Each gene seems to have its own distinct switch rate and potential follow up *var*. Mutual exclusive expression is regulated through several layers of epigenetic memory. While inactive *var* gene loci are clustered together with the telomeres in 6 – 8 foci at the nuclear periphery, a small subset of genes (including, but not restricted to, the one active gene) is moved away towards the euchromatic regions of DNA (Dzikowski et al., 2007; Freitas-Junior et al., 2000). Furthermore, active and inactive genomic loci have their own distinct pattern of histone modifications. Both H3K9me3 and H3K36me3 seem to be critical for correct multigene expression (Chookajorn et al., 2007; Lopez-Rubio et al., 2007). A switch from methylation to acetylation marks, especially at position H3K9, seems to be indicative of active *var* genes. Disrupting the function of histone methyltransferase PfSET2 leads to a loss of regulation (Jiang et al., 2013). Active *var*, especially if the genomic locus is in the subtelomeric region, are characterized by histone acetylation and an absence of the histone deacetylase PfSIR2 (Duraisingh et al., 2005; Freitas-Junior et al., 2005). Removing a *var* intron from its chromosomal context makes it transcriptionally active, while addition of the appropriate intron (termed promoter pairing) silences it again, suggesting crosstalk between those two elements (Calderwood, Gannoun-Zaki, Wellem, & Deitsch, 2003; Deitsch,

Calderwood, & Wellem, 2001). Furthermore, transcription by RNA Pol II is necessary for correct *var* gene regulation (Dzikowski & Deitsch, 2008). The C-terminal domain of RNA Pol II is not only subject to a vast array of post-translational modifications, it directly interacts with PfSET2 (Ukaegbu et al., 2014). Considering that disruption of this interaction leads to dysregulation of *var* gene expression, it can be hypothesised that RNA Pol II actively alters the histone landscape during transcription (Ukaegbu & Deitsch, 2015). The presence of a currently unknown, titratable factor has been shown when *var* promoters were expressed from episomal plasmids (Dzikowski et al., 2006). Increase of plasmid copy number changed expression of endogenous genes, transcending the *var* multigene family and affecting *stevor* and *rif* as well (Howitt et al., 2009).

Figure 2: Epigenetic control mechanisms of *var* gene expression.

Active and inactive *var* genes differ in a variety of epigenetic marks. While inactive *var* genes are located, together with the other telomeric regions of the genome, within the telomeric cluster, the active *var* gene and a small subset of inactive ones are moved towards euchromatic regions. The active locus however shows a distinct pattern of histone modification marks, most notably H3K4me3 and H3K9ac (instead of H3K9me3 and H3K36me3 at inactive genes). Histone tail modifying enzymes such as PfSET2 are recruited to active loci by RNA Pol II. Adapted from (Ay et al., 2015)

Despite all the knowledge about *var* gene regulation, the underlying mechanism of mutual exclusive control is still unknown. The parasite genome lacks the enzymes necessary for RNAi, therefore eliminating this mode of repression (Baum et al., 2006). Furthermore, the ability to activate large amounts of different *var* genes either through knockout of regulatory proteins or episomal promoter

expression seems to rule out the involvement of designated long-distance enhancers or strictly controlled subnuclear localisation (Dzikowski et al., 2007; Jiang et al., 2013; Voss et al., 2006). Another unanswered question is why only a small number of different *var* genes can be detected during infection (Montgomery et al., 2007). A mathematical model, which describes this phenomenon by assuming that every parasite in a population routinely reverts to a specific “steady-state” *var* gene, explains clinical symptoms but entirely lacks experimental evidence (Recker et al., 2011).

1.6. RIFIN

Even though RIFINs include more than 160 genes in the reference genome, information about their function is much more limited than PfEMP1. The proteins have a predicted size of 30-45kDa. Similar to PfEMP1, certain RIFINs are exposed on the surface of the infected red blood and mediate rosetting (Goel et al., 2015; Helmbj, Cavelier, Pettersson, & Wahlgren, 1993). It has been suggested before that each parasites expresses several *rif* genes at a given point in time (Petter et al., 2008). Phenotypic enrichment however leads to expression of only one *rif*, potentially hinting at mutual exclusive control within this family (Goel et al., 2015). *Rif* are not only expressed in ring stage parasites but can be found in merozoites as well as gametocytes (Petter et al., 2008; Petter et al., 2007). Similar to *stevor*, expression is significantly higher in field isolates than in cultured parasites and diminishes in splenectomised patients (Bachmann et al., 2009b; Fernandez, Hommel, Chen, Hagblom, & Wahlgren, 1999). Analogous to *var* genes, *rif* upstream promoter regions can be clustered into distinct groups as well. Specifically, A1, A2, AB, B and C. Experiments using episomally expressed *rifA1* promoters (which are oriented in a head to head manner to *var* gene clusters) show downregulation of *var* gene expression (Witmer et al., 2012). This finding would suggest crosstalk between the two multigene families beyond the previously mentioned titratable factor. Neither episomal expression of paired *rif* introns nor genomic integration of full, drug selectable 5' regions showed any degree of regulation within the *rif* multigene family (Araujo et al., 2018; Witmer et al., 2012).

Recently, RIFINs have been associated with direct host immune suppression through binding to leucocyte immunoglobulin-like receptor B1 (LILRB1) or leucocyte-associated immunoglobulin-like

receptor 1 (LAIR1) (Saito et al., 2017), making them the first example of *P. falciparum* small variant antigens that actively interfere with immune cells. RIFIN mediated rosetting is achieved by binding to blood group A antigen and glycophorin A (Goel et al., 2015). Considering that patients with non-O blood group have a higher likelihood of developing severe symptoms, RIFINs have been associated with disease outcome (Wahlgren et al., 1995).

The structure of RIFINs is conserved and includes, starting from the N-terminus, a PEXEL signal peptide, a semiconserved region, a hypervariable region, a transmembrane domain and a highly conserved intracellular c-terminus. RIFINs can be divided into two subgroups (A-type and B-type) depending on their structure. A-type RIFINs include a 25 amino acid long insert downstream of the PEXEL export motif within the semiconserved region (Joannin, Abhiman, Sonnhammer, & Wahlgren, 2008). While A-type RIFINs are being exported through the Maurer's Clefts to the surface of the infected red blood cell (Bachmann et al., 2015; Goel et al., 2015; S. A. Kyes, Rowe, Kriek, & Newbold, 1999), the majority of B-type RIFINs seem to remain within the parasite itself (Bultrini et al., 2009). A similar pattern can be observed in gametocytes with A-type RIFINs at the red blood cell membrane (even though surface exposure is uncertain) and B-types within the parasite. Furthermore, some evidence suggests that B-type RIFINs utilise a second transmembrane domain between the semiconserved and hypervariable region (Petter et al., 2007). A different study suggests that the predicted second transmembrane domain is in fact a dimerisation domain, which explains the dimer pattern seen in size-exclusion chromatograms of RIFINs (Goel et al., 2015). Another difference between A and B-type RIFINs is that the former ones are located at the apical tip of merozoites while the latter ones diffuse within the parasite (Bachmann et al., 2012; Petter et al., 2007).

1.7. STEVOR

With only 33 members in the reference genome (40 if pseudogenes are included), *stevors* are the smallest group among the VSAs (Gardner et al., 2002). Expression is restricted to 1-3 genes per parasite

and is elevated in field isolates (Blythe et al., 2008; Lavazec et al., 2007; Niang, Yam, & Preiser, 2009). In contrast to *rif*, the *stevor* multigene family showed an evolutionary diversification depending on the infected host, suggesting a recent switch in host cell ligand (Otto et al., 2018). Episomal expression of paired *stevor* promoters showed no change in endogenous gene expression, suggesting either a lack of co-regulation within the family or some promoter independent mechanism (Witmer et al., 2012). The predicted structure of the 35-40kDa proteins is highly similar to RIFINs - it includes (from the N-terminus) a signal peptide, a semiconserved region, a transmembrane domain, a hypervariable region, a second transmembrane domain and a conserved cytoplasmic tail (Cheng et al., 1998; Lavazec, Sanyal, & Templeton, 2006). Even though, considering the double transmembrane topology and the lack of the 25 amino acid insert, they resemble the structure of B-type RIFINs, the protein has been shown to be exposed on the surface of infected red blood cells where it localises to the base of knobs (Bachmann et al., 2015; Niang et al., 2014; Singh et al., 2017). STEVORs exposed on the surface seem to utilise only the second, C-terminal transmembrane domain and interact with glycophorin C (Bachmann et al., 2015; Niang et al., 2014; Niang et al., 2009). Unlike PfEMP1 and RIFIN, which are expressed slightly earlier, STEVOR only appears on the surface at trophozoite stage parasites. Another difference between STEVOR and B-type RIFINs is the localisation at the apical end of merozoites which resembles A-type RIFINs (Bachmann et al., 2012; Bachmann et al., 2015; Blythe et al., 2008; Khattab et al., 2008). Antibodies targeting STEVORs expressed in merozoites reduce invasion efficacy, thus proving exposure to the extracellular realm (Niang et al., 2014). The intracellular tail is interacting with spectrin and ankyrin of the red blood cell cytoskeleton in gametocytes, where it is exposed to the surface from stage III onwards. Presence of STEVOR decreases deformability of the infected red blood cell in a protein kinase A dependant manner, both in sexual as well as asexual stages (Naissant et al., 2016; Sanyal et al., 2012; Tiburcio et al., 2012). It has been suggested that the switch in deformability during gametocyte development is essential for maturation and release of the gametocyte into the blood stream (Tiburcio et al., 2012). Considering the importance for maturation, it has been suggested that one or several STEVORs might also be responsible for sequestration in the bone marrow. If a STEVOR

is indeed involved, it has not been identified in a recent small scale screen for gametocyte adhesion to human primary erythroblasts (Neveu et al., 2018).

1.8. Functional synergy between multigene families and their impact on immunity

Clinical isolates of *P. falciparum* show not only an increase in expression of multigene family members, they also bind to a wide range of surface receptors (Beeson et al., 1999; Blythe et al., 2008; Heddini et al., 2001; Ockenhouse et al., 1992; J. A. Rowe, Claessens, Corrigan, & Arman, 2009). It is therefore probable that these proteins are essential for parasite survival under *in vivo* conditions. Considering the large and diverse number of expressed proteins, synergistic interactions among them could be hypothesised. To date however, no study has shown direct interaction among VSA from different multigene families. They do follow, however, a distinct hierarchy of expression. *Var* genes are the first to be expressed, followed by *rif* (both of which are detectable on the surface of the infected red blood cell at an early trophozoite stage), and finally *stevor* (which appears from mid trophozoite stage) (Kaviratne, Khan, Jarra, & Preiser, 2002; S. Kyes, Pinches, & Newbold, 2000; S. A. Kyes et al., 1999). Considering that clumps of cells that result from rosetting would be recognised and cleared by the spleen, it seems that PfEMP1 mediated sequestration needs to preclude the presence of the sVSA on the surface. Furthermore, the increase in rigidity which is characteristic of later stage infected red blood cells and results in splenic clearance, is at least in part mediated by the presence of sVSA on the surface (Sanyal et al., 2012). It appears likely that the sequestration through PfEMP1 enables the emergence of rosetting. Another argument towards this theory is that red blood cell receptors utilised by PfEMP1 proteins, such as complement receptor 1, are also present on endothelial cells, opening up the possibility that PfEMP1 mediated rosetting is only a side effect (J. A. Rowe, Moulds, Newbold, & Miller, 1997).

It has been hypothesised that the presence of rosettes supplies merozoites with fresh red blood cells for invasion, a theory that is strengthened by the overlap of receptors utilised by invasion related

proteins and VSAs (Baum, Thomas, & Conway, 2003; Rydzak et al., 2015; Tham et al., 2010). Rosetting parasites have been associated with severe malaria in some, but not all studies and show a positive correlation with parasite growth (Carlson, Helmby, et al., 1990; Rogerson et al., 1999; A. Rowe, Obeiro, Newbold, & Marsh, 1995; J. A. Rowe, Obiero, Marsh, & Raza, 2002). Furthermore, rosettes protect parasites against invasion inhibiting antibodies while presence of rosette disrupting antibodies have been associated with positive disease outcome (Moll, Palmkvist, Ch'ng, Kiwuwa, & Wahlgren, 2015; Niang et al., 2014; J. A. Rowe et al., 2009). A strong argument for the importance of VSAs is the link between the presence of antibody recognition and protection (Abdel-Latif, Dietz, Issifou, Kremsner, & Klinkert, 2003; Carlson, Helmby, et al., 1990; Carlson, Holmquist, Taylor, Perlmann, & Wahlgren, 1990). Cross reactive antibodies against different PfEMP1 or RIFIN variants have been observed in immune patients and the presence of these antibodies protects against sequestration in experimentally infected monkeys (Abdel-Latif, Khattab, Lindenthal, Kremsner, & Klinkert, 2002; Baruch et al., 2002; Bengtsson et al., 2013; Tan et al., 2016). Recognition of both RIFIN as well as STEVOR semiconserved regions through patient antisera increases with age (Zhou et al., 2019).

1.9. *Plasmodium falciparum* protein export and host cell remodelling

In order for VSAs to interact with their host cell receptors, the protein needs to be exported out of the parasite, through the parasitophorous vacuole to the host cell membrane, where it is inserted and exported to the extracellular space. Furthermore, infected red blood cells generate exosomes and microvesicles which are involved in communication with both infected as well as uninfected red blood cells and endothelial cells (Mantel et al., 2016; Mantel et al., 2013; Regev-Rudzki et al., 2013). Considering that the red blood cell itself has no molecular machinery to perform such a task, the parasite has to construct the entire pathway on its own.

Proteins destined for export can be categorised depending on the presence (e.g. RIFIN, STEVOR) or absence (PfEMP1) of a '*Plasmodium* export element' (PEXEL) signal peptide (Hiller et al., 2004; Marti, Good, Rug, Knuepfer, & Cowman, 2004; Spielmann et al., 2006). Proteins designated for export are trafficked through the parasite plasma membrane either in vesicles or by membrane insertion followed

by extraction (Gruring et al., 2012; Mesen-Ramirez et al., 2016). Transport through the parasitophorous vacuolar membrane is facilitated exclusively by the PTEX- a 1.6mega Dalton protein complex which requires the cargo to be in an unfolded state (Ansorge, Benting, Bhakdi, & Lingelbach, 1996; de Koning-Ward et al., 2009; Gehde et al., 2009). After exiting the PTEX, cargo is transported by chaperones or protein complexes called J-dots (Knuepfer, Rug, Klonis, Tilley, & Cowman, 2005; Kulzer et al., 2012). The PTEX itself does not stretch out into the host cell cytoplasm and interacting partners on that side of the membrane are currently unknown (Ho et al., 2018). The exact mode of chaperone-based cargo retrieval is therefore unknown. Independent of transport mode, most proteins destined to reach the erythrocyte membrane (including PfEMP1) are first delivered to membranous compartments called Maurer's Clefts (Hanssen, Sougrat, et al., 2008; Lanzer, Wickert, Krohne, Vincensini, & Braun Breton, 2006; Tilley, Sougrat, Lithgow, & Hanssen, 2008). These closed membranous compartments are connected to the erythrocyte membrane by tubular structures, but not through direct membrane connections (Hanssen et al., 2010). Clefts are assembled soon after invasion and require several proteins for correct assembly (Gruring et al., 2011; McHugh et al., 2015; Rug et al., 2014). Notably, PfEMP1 trafficking protein 1 (PTP1) has been shown to be essential for J-dot mediated transport of PfEMP1 and STEVOR from the PVM (Rug et al., 2014). Imaging of Maurer's Clefts reveals budding vesicles which are partially coated (Hanssen et al., 2010). Both the coated vesicles as well as the connecting tubes are stained with PfEMP1 antibodies, suggesting a transport system where PfEMP1 is loaded into vesicles and then transported along the tubes to the red blood cell membrane (McMillan et al., 2013). As mentioned earlier, PfEMP1 is ultimately presented on membrane protrusions called knobs while STEVORs appear to be associated with the base of knobs (Singh et al., 2017; Waller et al., 2002). Knobs are the result of the spiral-like assembly of KAHRP underneath the erythrocyte membrane and the attachment of these complexes to the host cell cytoskeleton, which has undergone extensive modifications by the parasite (Chishti et al., 1994; Cyrklaff et al., 2011; Kats et al., 2014; Millholland et al., 2011; Watermeyer et al., 2016). The appearance of knobs on the surface of infected red blood cells leads to an increase in cell rigidity and stiffness, but their postulated role is solely in the

anchoring of PfEMP1. As has been established before, sequestration is essential for parasite survival in its mammalian host. Linkage of PfEMP1 to the host cell cytoskeleton appears to be necessary to withstand the strong forces that act on the cell during sequestration under blood flow (Y. Zhang et al., 2015).

Figure 3: Protein export in *P. falciparum*

Proteins designated for export are sorted at the endoplasmic reticulum, depending on the presence of a signal peptide (PEXEL or PNEP). During the co-translational import into the ER, the PEXEL motif (if present) is being cleaved. Proteins are transported in vesicles to the parasite plasma membrane, where they are released into the parasitophorous vacuole either through vesicle fusion with the plasma membrane or transporters. Proteins are exported into the host cytoplasm through the PTEX complex. Chaperones or J-dots transport the cargo further, either directly to the erythrocyte membrane or through the Maurer's Clefts, membranous parasite compartments within the host red blood cell. Adapted from (de Koning-Ward, Dixon, Tilley, & Gilson, 2016)

1.10. Research drive and rationale

Among the variant surface antigens, *var* have been the focus of research in the past. Most of the knowledge about regulatory mechanisms has been obtained by studying the mutual exclusive control of *var*. Moreover, most of the host receptors targeted by *P. falciparum* surface antigens have been associated with PfEMP1. The recent discovery of immune suppression by RIFIN signals that there is a lack in understanding about the role of small variant surface antigens in parasite – host interactions. Even more so, out of the three discussed multigene families, the *stevor* are the smallest and arguably least well understood one. They have been intensively studied by this lab before, generating most of the published information about these proteins. Despite those efforts, little is known about intracellular interaction partners, epigenetic regulation and host cell interactions. This work describes the development of a platform to study variant surface antigens in *P. falciparum* and how this platform was used to answer basic biological questions concerning STEVOR.

I. Identification of host receptors targeted by STEVOR

The study that identified glycophorin C as target of STEVOR utilized a small library of only three STEVOR (Niang et al., 2014). Within the *stevor* multigene family however exists a large diversity of different sequences. While the 3D7 reference genome includes 60 different *var* with numerous identified host ligands, only glycophorin C has been described so far for the 40 *stevor* in the 3D7 genome. The first part of this work describes two approaches to identify STEVOR with novel binding properties to red blood cells.

II. Development of a platform to study STEVOR in *P. falciparum*

In order to study STEVOR in *P. falciparum*, the protein can be either expressed from a plasmid or parasites with unique expression profiles can be obtained by limiting dilution single cell cloning. While the former entirely excludes epigenetic regulatory mechanisms, the latter only allows study of genes that are identified by chance instead of choice. This work will describe how a recently developed endogenous tagging method has been adapted to force expression of *stevor*, *rif* and *var* of choice under the control of their endogenous promoter.

III. Identification of intracellular interaction partners

The only study describing intracellular interaction partners of STEVOR to this day is restricted to the sexual stages of the parasite. Utilizing the parasite lines that express specific STEVOR of choice, co-immunoprecipitation and proximity labelling approaches will be utilized to identify the protein complexes that anchor STEVOR within the infected red blood cell.

IV. Functional evidence of *rif* immune suppression

While the main focus of this work lies on the *stevor* family, the possibility to force expression of any member of a multigene family of choice enables functional studies of PfEMP1 and RIFIN, expressed by *P. falciparum* under their endogenous promoter. The *rifin* knock-in cell lines generated in (II) force expression of genes that have previously been associated with immune evasion. Both cell lines will be tested for their ability to evade natural killer cells to provide functional prove that the knock-in method does not impede function.

2. Materials and Methods

2.1. *Plasmodium falciparum* cell culture

2.1.1. Culture conditions

Plasmodium falciparum parasites (3D7) were cultured as previously described (Trager & Jensen, 1976). Parasites were cultured in complete RPMI (RPMI 1640 medium supplemented with 0.5% AlbuMAX II, 25mM HEPES, 2g/l NaHCO₃, 50mg/l hypoxanthine and 10mg/l gentamicin) under microaerophilic conditions at 37 °C in human erythrocytes. In order to maintain a healthy parasite culture, haematocrit was maintained at 2.5% and parasitaemia was kept below 5% for culture maintenance. Health and parasitaemia of culture were controlled by Giemsa stained thin blood smears. Parasitaemia was raised to 8 – 9% if late stage parasites were to be harvested. Whole blood was donated by healthy non-malarial immune adult volunteers at the National University of Singapore Blood Bank or National Cancer Institute of Singapore. Informed consents were obtained from all donors in accordance with approved protocol and guidelines. Whole venous blood was collected in citrate-phosphate-dextrose-adenine-1.

2.1.2. Synchronization and parasite harvest

Parasites were synchronized with 5% D-sorbitol for 10 minutes at 37°C, which kills all stages except rings. Parasites were washed in iRPMI (RPMI 1640 medium supplemented with, 25mM HEPES, 2g/l NaHCO₃ and 50mg/l hypoxanthine). For tight synchronization, the same step was repeated 30 hours later, which results in a culture of only freshly re-invaded ring stages. Late stage parasites were enriched by centrifugation on a layer of 68% percoll (Rivadeneira, Wasserman, & Espinal, 1983). Non-rosetting parasites of mid-trophozoite stage or later remain on top of the percoll, while uninfected red blood cells and early stage parasites sink to the bottom.

2.1.3. Transfection and selection

Parasites were transfected as previously described (Fidock & Wellems, 1997). In short, 200µl of freshly re-invaded ring stage parasites at >10% parasitaemia are mixed with 340µl cytomix (120 mM KCl, 0.15

mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES) and 75µg of plasmid DNA. Mixture was transferred to 2mm electroporation cuvettes and transfected using a Bio-Rad Gene Pulser at 0.31 kV, 950 µF, and infinity resistance. Transfectants were selected with 4 nM WR99210 (Jacobus Pharmaceuticals). WR99210 resistant parasites were selected with 400 µg/ml G418 (Promega) at 1% parasitaemia for integration as previously described (Birnbaum et al., 2017). Genomic DNA was extracted (Macherey-Nagel) and integration PCR was performed using KOD Hot Start Polymerase (Novagen) as previously described (Birnbaum et al., 2017). A full list of primers used to verify integration can be found in table 1.

Gene ID	Fwd primer	Rev Primer
Pf3D7_0421300 (var)	GTATGTGTACATTTATGGCATAATGTAGGG	CCGACAAATATAACCACTATACAAAAAAAACACATG
Pf3D7_1101100 (rifin)	GGAAATTTTCGATCGTCAAACCTCAGAA	GGTACATTCAAACGAAAACACTACGTGTAC
Pf3D7_1254800 (rifin)	GTGCGAATTATATATGTCCAATTATGACAACGATCC	GAAAAACAGTACATGTACCAAACATCCTACC
Pf3D7_0102100 (stevor)	CGGTAGAAAAAGAATTATTGAAAACATATGAAGAAATATTTGATGAAG	GAAAAATATTATTCAAATAATGTTATATCACGTGTCAAACAAAATTAC
Pf3D7_0631900 (stevor)	CTAGGAAAATTATCAAATAACCATTAACATAAGGC	TTTCATCCAGAAAATACTATTCAAATAATAATATATCAGG
Pf3D7_1149900 (stevor)	CTCATTCAAACAAGACCAAAAGAGCGA	AATGTATATACGATTCACAAATTACATAATTATATTTTC
Universal plasmid primer	TAT TTA GGT GAC ACT ATA GAA TAC TCG	CTA CCT GCA CCT CCA GCA CCA GC

Table 1: Primers used to validate genomic integration of pSLI

2.1.4. Single cell cloning

Single cell clones of a mixed *P. falciparum* culture were obtained by limiting dilution (Rosario, 1981). Tightly synchronized parasites were placed on a shaker at schizont stage to facilitate single cell invasion. Four to six hours after invasion, parasitaemia was accurately counted (>2000 red blood cells counted). Parasitaemia should not exceed 5% and no more than 5% of infected cells should have multiple invasions (more than one parasite within one red blood cell). Cells are diluted to a final concentration of 0.5 parasites in 100µl culture medium with 5% haematocrit. Dilution is transferred to 96-well plate.

Medium is changed every 48 hours and culture was diluted 1:1 at day 6 to supply fresh red blood cells. Parasitaemia was controlled from day 12 onwards.

2.1.5. Induction of gametocytogenesis

Induction of gametocytogenesis was achieved by subjecting the parasite to stress conditions followed by N-acetylglucosamine treatment (Ponnudurai, Lensen, Meis, & Meuwissen, 1986). An initial culture of 2.5ml of packed infected red blood cells, 12 hours post invasion and freshly sorbitol treated, 3% parasitaemia, in 50ml of cRPMI in a T75 tissue culture flask was placed on a shaker to facilitate single cell invasion (day 1). Medium was changed daily for two days (days 2 and 3). On day four, parasitaemia was reduced back to 3% and 20% of spent medium was returned back to flask. Medium was changed daily on days five to ten with cRPMI + 10g/l N-acetylglucosamine. Gametocytes of stage three could be observed from day six onwards.

2.1.6. Parasite fractionation for western blot

Parasites were fractionated for western blot analysis using saponin as previously described with slight modifications (Ansorge et al., 1996). Selective solubilization of red blood cell membrane, Maurer's Clefts and the parasitophorous vacuolar membrane has been optimized and validated in our lab previously by Anthony Siao (manuscript in preparation). Percoll enriched late stage parasites were resuspended in 10x volume of 0.05% saponin in iRPMI and incubated at room temperature for 5 minutes. Insoluble fraction was separated from soluble by centrifugation at 900g for 5 minutes. Insoluble fraction was washed twice and resuspended in the same volume that was used for saponin lysis. Protein loading dye (6x Laemmli buffer) was added to both fractions and samples were boiled at 95°C for 5 minutes.

2.1.7. Surface trypsinization

Tryptic digest of surface exposed proteins was performed as previously described (Baruch et al., 1996). In short, percoll enriched parasites were incubated for 10 minutes with TPCK treated Trypsin (Sigma) at 37°C in iRPMI. Reaction was stopped by addition of cRPMI supplemented with 1mM

Phenylmethylsulfonyl fluoride (Sigma) and protease inhibitor cocktail (Nacalai Tesque). Parasites were washed twice with iRPMI supplemented with protease inhibitor cocktail. Samples of PfEMP1 knock-in parasites were sequentially extracted with 1% Triton X-100 (integral membrane proteins remain in the pellet) and 2% SDS (which solubilizes the membrane and releases integral membrane proteins to the supernatant). Samples of STEVOR knock-in parasites were extracted using RIPA lysis buffer (10x volume, 30 minutes, 4°C with Pierce Universal Nuclease for cell lysis (250U) and Nacalai Tesque protease inhibitor cocktail).

2.2. COS7 cell culture

2.2.1. Culture conditions

COS7 cells cultured according to manufacturers' instruction (ATCC). In short, cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-Glutamine (Invitrogen) and 2 mM Penicillin-Streptomycin (Invitrogen) at 5% CO₂. Upon >90% confluency, cells were dislodged from the tissue culture flask by treatment with 0.25% trypsin for 5 minutes at 37°C. Cells were washed with iRPMI and re-seeded at 10% confluency in a fresh flask or 6 well plate.

2.2.2. Transfection and red blood cell binding assay

COS7 cells were seeded into 6 well plates at 10% confluency. Once a confluency of 40-50% was reached, cells were transfected with 3µg of plasmid DNA using Lipofectamine 2000 according to manufacturers' protocol. Red blood cell binding assay was performed two days later. COS7 cells were incubated with red blood cells resuspended in 200 µl iRPMI with 20 fetal calf serum at 10% haematocrit for 2 hours at 37°C. Unbound red blood cells were washed away with PBS and rosettes were observed by light microscopy using a 20x objective. At least 1000 COS7 cells were counted, scoring cells with at least three bound red blood cells as rosette.

2.3. Cloning

All PCR amplifications were performed using KOD Hot Start Polymerase (Novagen). Cloning inserts were amplified from 3D7 cDNA (pDisplay and pET24a) or gDNA (pSLI). Templates for the two new pSLI

tags 3.HA and c-myc.BioID were kindly provided by Dr. Omar Sheriff. Correct size of PCR products was controlled by agarose electrophoresis. Products were purified for digest using QIAquick PCR Purification Kit (Qiagen). A full list of primers used to amplify fragments by PCR can be found in table 2.

To exchange the tag of the existing pSLI.spacer.2xFKBP.GFP.T2A.NeoR/hDHFR (kindly provided by Tobias Spielmann), the plasmid was digested with FastDigest Pfl23II and FastDigest Sall (ThermoScientific). Tags were inserted using NEBuilder® HiFi DNA Assembly Cloning Kit (NEB) according to manufacturers' protocol.

Gene inserts and their respective plasmid backbones were digested with either XmaI and Sall-HF (NEB, pDisplay), NheI and XhoI (NEB, pET24a) or NotI and MluI (ThermoScientific, pSLI). Plasmid digest was run a 1% agarose gel and the band corresponding to the fully digested plasmid extracted using NucleoSpin™ Gel and PCR Clean-up Kit (Macherey-Nagel). PCR was purified using QIAquick PCR Purification Kit (Qiagen). Ligation was performed with T4 DNA Ligase (2x rapid ligation buffer, Promega) and transformed into XL10-Gold ultracompetent E. coli cells (Stratagene) via heat shock treatment and selected with 100µg/ml Ampicillin. Resulting clones were verified by sequencing (1st BASE).

Gene ID	Target plasmids	Forward primer	Reverse Primer
PF3D7_0101800	pDisplay	ACACCCGGGCAAAGAACAACGAT AAATTCAAG	TGTGTCGACTAAATGTTTTAAATA CTTATCTCTTCC
PF3D7_0222800	pDisplay	ACACCCGGGAAGACCAAAAGAA CAACGATAAATTCAAGA	TGTGTCGACTAAGTGTTTTAAATA TTTATCTTTTCC
PF3D7_0425500	pDisplay	ACACCCGGGAGTCTCATAAACAA TTACACCCAAAG	TGTGTCGACACCTGTTTTAAATT ATCTAAATAATTATCATGTAC
PF3D7_0700400	pDisplay	ACACCCGGGAAAAGAACAACGA TAAAATCAAGACTTTTAG	TGTGTCGACTAGGTGTTTTAAAT ACTTATCTCTTCC
PF3D7_0832600	pDisplay	ACACCCGGGAACAAAAATATAA AAATATCTTATATGTTCC	TGTGTCGACACCCGATTTTAAATT ATCTAA
PF3D7_1149900	pDisplay	ACACCCGGGAAAAGAGCGACGA TAAAATCAAGAC	TGTGTCGACTAAGTGTTTTAAATA CTTATCTCTTCC
PF3D7_1254100	pDisplay	ACACCCGGGACGATAAATTCAAG ATTATTAGCACAAACAAA	TGTGTCGACTAGTTTTTTTAAATA CTTATCTCTTCC
PF3D7_1372800	pDisplay	ACACCCGGGAATAACACCAAAAG AACAACGATACATTC	TGTGTCGACTCTCCCTTTTAAAGTG TTTTAAATAC
PF3D7_1400700	pDisplay	ACACCCGGGAAAAGCCTCTTACA AAAGAACAC	TGTGTCGACTAAGTGTTTTAAATA CTTATCTTTTCC

PF3D7_0222800	pET24a	GTGCTCGAGTAAGTGTTTTAAAT ATTTATCTTTTCCT	ATGGCTAGCAAGACCAAAAGAAC AACGATAAATTCAA
PF3D7_0832600	pET24a	GTGCTCGAGACCCGATTTTAAATT ATCTAAATAATT	TATGGCTAGCAACAAAAAATATA AAAATATCTTATATGTTCCAA
PF3D7_1400700	pET24a	GTGCTCGAGTAAGTGTTTTAAAT ACTTATCTTTTCC	ATGGCTAGCAAAAGCCTCTTACA AAAGAACACAC
## 3xHA	pSLI	GGGCGTACGTACCCATACGACGT C	GGGGTTCGACGGCATAATCTGGA AC
## c-myc.BioID	pSLI	TGCTGGAGGTGCAGGTAGACGT ACGGAACAAAACTCATCTCAGA AGAG	AACTTCCTCTTCTTCTCCGTCGA CTTCAAGCTTCTCTGCGCTTC
Pf3D7_0421300 (var)	pSLI	CTCGCGGCCGCTAACACAACCGA ATACTTTATATTTTAAAT	AGCACGCGTTATATCCCACACAT CTCCTATAGGATAT
Pf3D7_1101100 (rifin)	pSLI	CTCGCGGCCGCTAACGCCAAAAA TGTAAGATAAATGTG	AGCACGCGTTTCTTCTAATAATTT TATATATTGGAG
Pf3D7_1254800 (rifin)	pSLI	CTCGCGGCCGCTAATGATGCAAC AATTTTCATG	AGCACGCGTTTCTTTAATAATTT TATATATTGGAG
Pf3D7_0102100 (stevor)	pSLI	CTCGCGGCCGCTAAGGAAATGTC TACGGTAGAAAAAGAATTATTG	AGCACGCGTGGTGCATAAATGTT TCTTGCAATCATGTTTCCATG
Pf3D7_0631900 (stevor)	pSLI	CTCGCGGCCGCTAAGCACAAACC CAAAACCATAATCC	AGCACGCGTCTTACATAAATGTTT CTTGCAATCATGTTTCCATG
Pf3D7_1149900 (stevor)	pSLI	CTCGCGGCCGCTAAGCACAAACC CAAAACCATAATCCGC	AGCACGCGTCTTACATAAATGTTT CTTGCAATCATGTTTCCATG

Table 2: Primers used to clone inserts and tags into pDisplay, pET24a and pSLI plasmids

2.4. STEVOR purification and red blood cell binding

2.4.1. Expression and purification of recombinant protein

Rosetta(DE3) cells were transformed with pET24a plasmids via heat shock treatment. Cells were grown in Lysogeny broth containing 100µg/ml ampicillin overnight at 37°C. Next morning, cells were diluted in fresh Lysogeny Broth and recovered until an optical density at 600nm wavelength (OD₆₀₀) of 0.2 was reached. Cells were transferred to 30°C and expression was induced by addition of 200µM (pET24a.Pf3D7_0222800SC and pET24a.Pf3D7_1400700SC) or 400µM (pET24a.Pf3D7_0832600) IPTG (Isopropyl-β-D-thiogalactopyranosid) for 6hours. Bacteria were harvested by centrifugation and frozen at -80°C.

Pellet was resuspended in binding buffer (50mM NaH₂PO₄, 0.5M NaCl, 10µM imidazole, pH8 + Pierce protease inhibitor cocktail) and lysed by sonication. Lysates were cleared by centrifugation and incubated with Ni-NTA beads for 1hr at 4°C with gentle rocking. Lysate was removed and beads were

washed 5 times with wash buffer (50mM NaH₂PO₄, 0.5M NaCl, 33.5μM imidazole, pH8 + Pierce protease inhibitor cocktail). Bound protein was eluted twice in elution buffer (50mM NaH₂PO₄, 0.5M NaCl, 250μM imidazole, pH8 + Pierce protease inhibitor cocktail).

2.4.2. Red blood cell binding assay

Per reaction, 50μl of red blood cells were pre-treated with either Trypsin (2mg/ml), Chymotrypsin (2mg/ml), Neuraminidase (0.1Units/ml) or without enzyme in 500μl of iRPMI for 2hrs at 37°C. Red blood cells were washed thrice with iRPMI to remove enzyme. Purified protein was incubated with the pre-treated red blood cells in a total volume of 600μl of iRPMI with 20% fetal calf serum for 2hrs at 37°C with gentle shaking. Cell suspension was overlaid with 500μl of DBP (Dibutyl phthalate) oil. After centrifugation, the red blood cells remain at the bottom of the tube while the oil replaces the iRPMI including unbound protein, thus removing unbound protein from the red blood cells. The cell pellet is resuspended in 0.5M NaCl for 15 minutes to elute membrane bound proteins. After another centrifugation, the NaCl solution with eluted protein is collected and boiled together with 6x protein loading dye for 5 minutes at 95°C. Eluted protein was detected by western blotting probing for the 6xHis tag of the purified protein.

2.5. qRT-PCR

RNA was extracted from highly synchronous parasites using TRIzol reagent according to manufacturers' protocol. Residual DNA was removed with RQ1 RNase-Free DNase (Promega). RNA was purified with RNeasy Mini Kit (Qiagen) and cDNA synthesized with SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturers' manual using a mix of Oligo-dT (25-mer) and random primer (9-mer).

Unique primer pairs to a subset of *var*, *stevor* and *rifin* genes were designed and their efficacy tested against *P. falciparum* genomic DNA. All primer pairs showing an amplification efficacy of between 80 and 110% were deemed appropriate for further use. Quantitative RT-PCR reactions were performed using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems) according to manufacturers' protocol and analysed using an Applied Biosystems 7500 Fast Real-Time PCR System. Each biological replicate was

run twice on the same 96 well plate and the average of the two was calculated. Ct values were normalized to PF3D7_0717700 (serine tRNA ligase) and relative expression calculated ($2^{-\Delta Ct}$). A full list of primers used can be found in table 3.

Gene ID	Fwd primer	Rev primer
Pf3D7_0115700 (var)	AATATTGCGATCTTAATAGGTATGATTGCG	GCACCACCTGTTATTCGCTTTTG
Pf3D7_0421100 (var)	GGAAAATATCAACTAACTAATCATGG	GCATTCCTTAATTTATAATAATTTGGATC
Pf3D7_0421300 (var)	GCTAACCAATATAAAGATGACTTGAAAGG	AAAACGCTCTACATCGTTTCCG
Pf3D7_0533100 (var)	TGGCGAAGGTATAATGATAAAGG	TACTGTACTACATTCAGATATTTGTCC
Pf3D7_0617400 (var)	CATACAGTAGTGCCAAGGACC	GCGCAAGGATTATTTTTATCAAAACC
Pf3D7_0632500 (var)	CGCTGGATGAAAGTGTCAAAG	TAACAAGGATTCTCTTTACCACC
Pf3D7_0421100 (var)	GGAAAATATCAACTAACTAATCATGG	GCATTCCTTAATTTATAATAATTTGGATC
Pf3D7_0421200 (rifin)	ATGTCACAAAGATTGCTCG	GCAATGCTGTTTTTATAGCTT
Pf3D7_0421500 (rifin)	CACCAACTACTAAGTCACGATT	ATGCCTCTAACTTTTCTGTTAATTCC
Pf3D7_0532900 (rifin)	CGAAATGAACCATCACAACACA	CTGTTTTGTTCACACGTTTCATTG
Pf3D7_1101100 (rifin)	AATTATACGCACCATCTAACTATGAC	CATTTTGTATATCAGTTTGTAACTTAAG A
Pf3D7_1149800 (rifin)	CCATACATCTCACCACATACAC	CAATAATTTTTTTATATCTTTGTCACATT GTTC
Pf3D7_1254800 (rifin)	CGCGACATTGAACACTAATATACTAATG AG	TCAATTGTTTTCGCAATAAATGCGTTG
Pf3D7_0421200 (rifin)	ATGTCACAAAGATTGCTCG	GCAATGCTGTTTTTATAGCTT
Pf3D7_0101800 (stevor)	TGATGCCCTTGCTAGTTATGC	CAGAAGTTGCAGCAGGTACTGT
PF3D7_0102100 (stevor)	GGTTTGCAAAGGCCAAAATA	CAAGCTGAACTACCAGCTTCAA
PF3D7_0114600 (stevor)	CAGCTATTCAAGCAGGTGCTAA	CAATACCACAACCTCCAGGAA
Pf3D7_0115400 (stevor)	GGCTGCCTTTGATACCTTGA	TGTACCGCCTGCAAACATAG
PF3D7_0200400 (stevor)	GCCCTAAGTGCTGTTGCTTC	CACCAACAACACATGCTTTCA
PF3D7_0200900 (stevor)	CTGCTGCCATTGCTACCTTT	AGCAGCACCAGATGCACATA
PF3D7_0201300 (stevor)	GCAAAATCTGCTGCCCTTAC	ACCATTGTCACAGGTTCCAC

PF3D7_0221400 (stevor)	TAAATTTACGAAGGCGCTTG	CCAGAGCTGCAATACCATAA
PF3D7_0222800 (stevor)	TGCTGCTGTCACTTCTAGCTTT	CTACACCTCCTGCTGCAGTAAC
PF3D7_0300400 (stevor)	AAAGGTGCTGCTATTTCTACCG	CAGCAGCACAACTACTGTACC
PF3D7_0324600 (stevor)	TGCATGCTGCTAAAGTTGCT	TTGGTTGCACAAAGAACTGACT
PF3D7_0400800 (stevor)	TGGCAGCTACCAAAGCTACA	TCAGGACCCCAAGCTGTAATA
PF3D7_0401500 (stevor)	AAATGCGCATCCTCTATCACT	AGTTCCGACCCCATCACTAA
PF3D7_0402600 (stevor)	TTTATGGTATTAGCGCTGCAAG	GCGCTTTCTATTGATGTTTGAG
PF3D7_0425500 (stevor)	TCATCATCCCAATTGACGAA	CTTCATATTCGACGGACGTT
PF3D7_0425500 (stevor)	AATGCGTTGCCCTCTATATC	TCATATTCGACGGACGTTAT
PF3D7_0500600 (stevor)	GCAAAGGTTCTTGCTGGTGT	GCTATTCTCATAGCATCCCAATG
PF3D7_0617600 (stevor)	CTGCACCTTATGATGCTTGTGT	AAACAGTTCCGGCCATATCA
Pf3D7_0631900 (stevor)	GTTGATGCCATCCTTCCTGT	ACGCTTCGGTTGCTTTAAGA
Pf3D7_0700400 (stevor)	GCAAAAGCTGCTATCCTTGG	ACACCACCTGATGCTAAGT
PF3D7_0700700 (stevor)	CCTGTTGGTTTATGGTCTCC	ATGTTTTCGTCTGTTGCGTA
PF3D7_0732000 (stevor)	TTGCAGAAGTGCCTAAGAATTG	GCACTAACAGTAGTTGCTTCTCCT
PF3D7_0832000 (stevor)	ACAGAATTCGCTGGTAATGT	AACTTGACAGCAAAGAACTC
PF3D7_0832400 (stevor)	AAGTGAGATGTTCCCGTGGT	TGGACAGTAAAGTGCACAAG
PF3D7_0832600 (stevor)	AATTGTCCCAAACCCTTGTC	AGCCTTCGTAGCCATTGAAC
PF3D7_0832900 (stevor)	TACGTGCGCATCCCATATTA	TTCCGACTAAATCACCAGCA
PF3D7_0900900 (stevor)	TTGCAGAAGTGCCTAAGAATTG	CATAGCAGCACTTGACAGAG
PF3D7_0901600 (stevor)	GGCTGCACTTGCTTACTTTTC	TAGCACATGTTGCACCTCCT
PF3D7_1000800 (stevor)	TGGGGCTCCTACATTAAGTCA	CACGACAATTGCCATACCAT
PF3D7_1040200 (stevor)	AGGTGCTCTTGCTGAGTATGC	CAAATTCAGTGCCTGCTTG
PF3D7_1100700 (stevor)	AGAACGTTGTGTTGGAGGTGT	GGAAGCCATAAACCAACAGG
Pf3D7_1149900 (stevor)	TGACGTTGCTGCCTGAATA	ACCAACGCCTGCTTGAATAG

PF3D7_1254100 (stevor)	TGCGTGCAAATCCTCTATCA	AAACAAGCTGTACTACCTGC
PF3D7_1254300 (stevor)	GCTAAAACGGCTGCCCTAA	CACCACTTGACAAAAATCCA
PF3D7_1254600 (stevor)	TCGGGCATGAGTCCCAATT	TTAGGGCAGCCGTTTATGCA
PF3D7_1300900 (stevor)	GCCGTTGCTTCTCTGTTTATT	ATCAGTAGCACCGGCAAGAG
PF3D7_1372500 (stevor)	CCTTACTAAATTGACCCAAGC	CTTTTCTTCTTTTACGCAGC
PF3D7_1372800 (stevor)	CTGCTAAAACGTGTGCGTCT	ATTTCAGCAGGCAAACTA
PF3D7_1400700 (stevor)	CTCCGTTGGACCACCTTAT	CTTGCAGCAGTTCCGGTTAT
Pf3D7_1479500 (stevor)	GCAAAAGTTGCTGTCATTGG	GAGCCTGCTGCATTAAGTGA
PF3D7_1479900 (stevor)	CATGAAGGCTGTTGCTGATTAT	ATCAGTAGCACCTCAACACAT
PF3D7_0717700 (serine tRNA ligase)	AAGTAGCAGGTCATCGTGGTT	TTCGGCACATTCTCCATAA
PF3D7_0102100 Short (stevor)	TAATGGTCAAAATAACAATTATAATATAA GCGTC	GGCATAATCTGGAACATCGTAAGG
PF3D7_0102100 Long (stevor)	GTGCATTCTTAACAATCTTAGGTTTGG	GGCATAATCTGGAACATCGTAAGG

Table 3: List of primers used for qRT-PCR

2.6. Western Blot

Protein samples were separated in 12% SDS-polyacrylamide gels and transferred to low fluorescence PVDF membranes (BioRad). Immunoblots were blocked with Odyssey Blocking Buffer (PBS base) (LiCor) for 1hr at room temperature. Primary antibodies were incubated either 1hr at room temperature or overnight at 4°C in blocking buffer. After three washes with PBS and 0.1% Tween-20 (Sigma), appropriate secondary antibodies were incubated for 1hr at room temperature in blocking buffer with final concentrations of 0.1% Tween-20 (Sigma) and 0.05% Sodium dodecyl sulfate (1st Base). Membrane was washed three more times with washing buffer and once with PBS. Membranes were scanned with a LiCor Odyssey CLx imager. Relative intensities were measured with Image Studio Lite Ver 5.2. Dilution of antibodies can be found in table 4.

Name	Supplier	Dilution Western Blot	Dilution Immunofluorescence analysis
Primary antibodies			
Mouse α GFP	Roche	1:1000	1:200
Rat α HA	Roche	1:3500	1:200
Mouse α c-myc	Sigma	/	1:500
Rabbit α c-myc	Cell Signaling Technology	1:1000	/
Rabbit α sEMP1	GenScript	1:3500	1:500
Rabbit α EXP2	GenScript	1:3500	1:500
Rabbit α Aldolase	GenScript	1:3500	/
Rabbit α GBP130	GenScript	1:3500	/
Rabbit α GapM	(Rayavara et al., 2009)	/	1:45000
Rabbit α Gap45	(Green et al., 2008)	/	1:45000
Mause α Spectrin- α 1	Abcam	1:3500	/
Rabbit α Glycophorin C	ProteinTech	1:3500	/
Rabbit α Histone3	Abcam	1:10000	/
Mouse α 6-His	Pierce	1:1000	/
Secondary antibodies			
IRDye800CW α rabbit	LiCor	1:10000	/
IRDye800CW α mouse	LiCor	1:10000	/
IRDye800CW α rat	LiCor	1:10000	/
IRDye680RD α rabbit	LiCor	1:5000	/
DyLight594 α mouse	Jackson ImmunoResearch	/	1:1000
DyLight594 α rat	Jackson ImmunoResearch	/	1:1000
DyLight488 α rabbit	Jackson ImmunoResearch	/	1:1000

Table 4: List of antibodies used for western blot and immunofluorescence analysis

2.7. Immunofluorescence analysis

Thin blood smears of synchronous parasite cultures (intracellular stages) or percoll enriched schizonts (merozoites) were fixed either with ice cold methanol for 10 minutes (intracellular stages) or airdried (merozoites). Airdried slides were fixed with 4% paraformaldehyde (Sigma) for 5 minutes at room temperature followed by membrane permeabilization using 0.01% saponin (Sigma) for 5 minutes at

room temperature. Slides were blocked with 1% bovine serum albumin (Sigma) in PBS with 1% Tween-20 (Sigma). Primary antibodies were incubated for 1hr at room temperature in blocking buffer. After two washes with PBS with 1% Tween-20, appropriate secondary antibodies and Hoechst 33342 (1 μ M) were incubated for 1hr at room temperature. After another two washes slides were mounted with Fluoromount G (SouthernBiotech). Slides were visualized on a Nikon Eclipse Ti fluorescent microscope with a Nikon Plan Apochromat Lambda 100X Oil objective. Pictures were taken using an Andor Zyla sCMOS camera and analysed using ImageJ 1.52h. Dilution of antibodies can be found in table 4.

2.8. Microarray

Total RNA was isolated from highly synchronized *P. falciparum* ring- and trophozoite-stage parasites (18, 24 and 30hpi) and hybridized against a reference pool of 3D7 cultures as previously described (Bozdech et al., 2003). A detailed step by step protocol has been published elsewhere (Bozdech, Mok, & Gupta, 2013).

In short, parasites were harvested, lysed in TRIzol and snap-frozen in liquid nitrogen until further use. RNA was isolated by removal of proteins and DNA using chloroform. RNA was precipitated using isopropanol and washed in 70% ethanol. Complement DNA was synthesized with SuperScript II Reverse Transcriptase (Invitrogen) using a mix of Oligo-dT (25-mer) and random primer (9-mer). DNA was further amplified using the SMART PCR method which uses the template-switching mechanism of the reverse transcriptase enzyme and thus ensures the synthesis of full-length mRNA transcripts from both 5' and 3' ends. Product was cleaned up using the MinElute PCR Purification Kit (Qiagen), followed by labelling of sample cDNA and pool cDNA with Cy5 and Cy3 respectively. Microarray chips were kindly provided by the Bozdech, Z. lab. Scanning and image acquisition of the hybridized microarray chips was performed using the PowerScannerTM (Tecan) at 10 μ M resolution under automated photomultiplier (PMT) balancing. Hybridized spots were analysed and pre-processed using GenePix[®] Pro Microarray Image Analysis Software v6.0 (Axon Instruments). Features with flag>0 and with median foreground intensity greater than 1.5fold median background intensity for either channel passed quality control and were accepted. Local background correction using normexp, followed by lowess

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normalization across all features were carried out to generate the normalized Log2 gene expression ratios.

2.9. STEVOR precipitation

2.9.1. Anti-HA co-immunoprecipitation

An equivalent of 100µl of percoll enriched late stage parasites (volume was adjusted according to final parasitaemia) were incubated with 20mM dithiobis(succinimidyl propionate) (DSP) in PBS (10x volume of parasite pellet) for 30 minutes. The reaction was quenched with 25mM Tris for 10 minutes. Parasite pellets were snap-frozen in liquid nitrogen and stored at -80°C until further use. Parasites were lysed in 10x volume of RIPA buffer with Pierce Universal Nuclease for cell lysis (250U) and Nacalai Tesque protease inhibitor cocktail for 30 minutes at 4°C. Insoluble debris was removed by centrifugation. The soluble fraction was split into two equal fractions and incubated with 50µg of either Pierce Anti-HA Magnetic Beads or Pierce Anti-c-Myc Magnetic Beads overnight at 4°C with gentle rotation. Lysate was removed the next day and beads were washed 5 times for 5 minutes at 4°C with 1ml of RIPA buffer.

2.9.2. Streptavidin precipitation

3D7 and 1149900.c-myc.BioID parasites were sorbitol synchronized at mid ring stage and cultured in cRPMI supplemented with additional 50µM biotin for 24 hours. An equivalent of 100µl of percoll enriched late stage parasites (volume was adjusted according to final parasitaemia) was snap frozen in liquid nitrogen and stored at -80°C until further used. Parasites were lysed in 10x volume of RIPA buffer with Pierce Universal Nuclease for cell lysis (250U) and Nacalai Tesque protease inhibitor cocktail for 30 minutes at 4°C. Insoluble debris was removed by centrifugation. To remove excess of soluble biotin, the soluble fractions were dialysed using Slide-A-Lyzer cassettes (2000 Dalton molecular cut-off) (2x 4hrs followed by a third overnight step). Dialysed lysates were incubated with 100 µg of pre blocked (Odyssey Blocking Buffer (PBS base) (LiCor), 1hr at room temperature) Pierce Streptavidin Magnetic Beads overnight at 4°C with gentle rotation. Lysate was removed the next day and beads were washed thrice in SDS wash buffer (2% SDS), twice in high salt RIPA (RIPA supplemented with

350mM NaCl), thrice in Urea wash buffer (50mM Tris-HCl, 50mM NaCl, 3M urea, Ph 7.6) and twice in RIPA (1ml, 5 minutes at room temperature each wash).

2.9.3. On-bead digest and mass spectrometric analysis

All precipitations for mass-spectrometric analysis were performed identically. Beads were washed five times in 100mM NH_4HCO_3 . Beads were finally resuspended in 100 μl of 100mM NH_4HCO_3 . Thirty μl of 10mM DTT are added to each sample and incubated for 1hr at 56°C. Three μl of 550mM IAA (Indole-3-acetic acid) are added to each sample and incubated for 45 minutes at room temperature in the dark. One μg of Pierce Trypsin Protease, MS Grade was added and incubated overnight at room temperature in the dark. The supernatant was desalted using Pierce Peptide Desalting Spin Columns according to manufacturers' protocol. The final elution was highly concentrated (almost dried) using a SpeedVac Vacuum Concentrator and resuspended in 10 μl 0.1% formic acid. Mass spectrometric analysis was performed by Ameya Sinha at MIT, Boston and Proteos, Singapore.

2.9.4. Mass Spectrometric analysis

Peptides were separated by reverse phase HPLC (Thermo Easy nLC1000) using either a self-packed 15cm Picofrit column (New England Objective) or an EASY-Spray column, 50 cm x 75 μm ID, PepMap RSLC C18, 2 μm (Thermo Fischer ES803A) over a 120 minute or a 90 minute gradient before nanoelectrospray using a Q-Exactive HF-X mass spectrometer (Thermo Scientific). The mass spectrometer was operated in a data-dependent mode. The parameters for the full scan MS were: resolution of 60,000 across 350–1500 m/z, AGC 1e^3 and maximum injection time (IT) 50 ms. The full MS scan was followed by MS/MS for the top 15 precursor ions in each cycle with a NCE of 28 and dynamic exclusion of 30 s and maximum IT of 300 ms. Raw mass spectral data files (.raw) were searched using Proteome Discoverer (Thermo Fisher) and Mascot or SEQUEST (Eng, McCormack, & Yates, 1994; Perkins, Pappin, Creasy, & Cottrell, 1999). Search parameters were: 10 ppm mass tolerance for precursor ions; 0.8Da for fragment ion mass tolerance; two missed cleavages of trypsin; fixed modification was carbamidomethylation of cysteine; variable modifications were methionine

oxidation, and serine, threonine and tyrosine phosphorylation using the *Plasmodium falciparum* reference proteome from Uniprot (<https://www.uniprot.org/proteomes/UP000001450>). Only peptides with a Sequest Xcorr score greater than or equal to 2 or a Mascot score greater than or equal to 25 and an isolation interference less than or equal to 30 were included in the data analysis.

2.10. Natural Killer cell assay

Natural Killer cell killing assay was performed as previously described (Ye et al., 2018). Briefly, natural killer cells (CD56+CD3-) were obtained from peripheral mononuclear cells or patient buffy coat by negative selection using EasySep™ Human NK Cell Enrichment Kit (Stemcell Technologies). Peripheral blood mononucleated cells were purified from whole blood. To this end, blood was diluted with an equal volume of 2mM EDTA-PBS and PBMCs isolated over a Ficoll-Paque PLUS (GE Healthcare) density gradient. Contaminating red blood cells were lysed with ACK lysis buffer (Life Technologies). Synchronized schizont stage parasites at a parasitaemia of 0.5% were incubated with natural killer cells at a ratio of 1:10 for 96 hours in cRPMI. Parasitaemia was quantified by flow cytometry and Giemsa-staining. Parasite DNA was stained using Hoechst 33342 (20 µg/ml) (Thermo Scientific). Calculation for reduction in parasitemia is as follows:

$$\% \text{ Parasitemia reduction} = \frac{\text{Parasitemia}_{iRBC} - \text{Parasitemia}_{NK+iRBC}}{\text{Parasitemia}_{iRBC}} \times 100$$

3. Results

3.1. Identification of STEVOR with novel erythrocyte binding properties

As described earlier, both PfEMP1 as well as RIFIN interact with several ligands on the surface of both human red blood cells as well as endothelial cells. STEVORs on the other hand have only been associated with glycophorin C binding (Niang et al., 2014). Considering the diversity displayed within the STEVOR multigene family, interaction with other, so far unidentified surface ligands appears possible. This chapter discusses the research done to identify further potential interaction partners.

The diversity within the *stevor* multigene family has been described before (Blythe et al., 2009). According to the previous clustering of the amino acid sequence of *stevor* from different laboratory lines and field isolates, the semiconserved regions group into three distinct clusters while the hypervariable region shows no clustering at all. These findings highlighted the predicted role of the semiconserved region in potential host ligand interaction while the hypervariable region was predicted to be involved in immune evasion. Another recent report suggested the presence of a group of *rif* which appears to link the *stevor* and *rif* multigene families (Otto et al., 2018). In order to explore the diversity within the *stevor* family and the relation of *stevor* to *rif*, phylograms of the 3D7 STEVOR and RIFIN were drawn and grouped into potentially functional divergent groups.

3.1.1. Sequence alignment highlights diversity within STEVOR multigene family

The amino acid sequences of all STEVOR in the 3D7 reference genome were aligned using ClustalX2.1 and the resulting phylograms drawn with Tree Of Life (Figure 4) (Ciccarelli et al., 2006; Higgins & Sharp, 1988).

The resulting tree splits into several subgroups of *stevor*. While most of them show little divergence, as is indicated by the short branch length towards the root of the tree, one branch deviates from the rest and encompasses genes with an overall higher diversity from the rest. Based on this observation, *stevor* can be grouped into two clades A and B. The two clades separate from each other relatively late after several common branching points indicating a common ancestor and a recent split of the two

groups. Notably, the glycoporphin C binding protein Pf3D7_0102100, seems to be an intermediate between the two groups. Clade A, which consists of 75% of all *stevor* and includes all three glycoporphin C binding proteins, displays an overall higher degree of similarity (Niang et al., 2014). This is further highlighted by the appearance of glycoporphin C binding proteins (indicated with black arrows) among different subgroups within this Clade. The second clade B shows an overall longer branch length and therefore higher diversity compared to the rest of the proteins. Evolutionary, the *stevor* genes showed a strong diversification between gorilla and human infecting parasites, resulting in a distinct split within this multigene family (Otto et al., 2018). This split could explain the division observed in the tree.

The previously generated tree comparing the amino acid sequence of the semiconserved region of STEVOR from different laboratory strains and field isolates clustered into two main branches (Blythe et al., 2009). Most of the 3D7 *stevor* (PF3D7_1479500 and PF3D7_0732000 being the exception) grouped into one of them. The large difference between the two trees arises from two factors. The previously generated tree is based only on the semiconserved region of the protein, excluding two thirds of the sequence from the alignment. Furthermore, it compares genes found in several different strains from different endemic regions. This resulted in preferential clustering of genes from the same endemic regions (with few exceptions). Taken this into account, it appears as if the diversity described before rather highlights regional clusters than functionally diverse groups.

Figure 4: Alignment of the amino acid sequence of all 40 *stevor* genes

Alignment of all 40 annotated *stevor* groups the genes into two clades A and B. While Clade A displays an overall higher degree of conservation, it includes all 3 glycoporphin C binding proteins (indicated with black arrows). Clade B diverges from the rest as is indicated by the longer branch lengths. The glycoporphin C binding protein Pf3D7_0102100 appears to be an intermediate between the groups, indicating that one of the clades evolved from the other.

3.1.2. Subgroup of STEVOR and RIFIN converge towards each other

Considering the structural similarities between STEVOR and RIFIN, the amino acid sequences of both families were aligned and a tree drawn (Figure 5). Comparing the sequences from all small variant

surface antigens, STEVOR and RIFIN form distinct clusters without overlap (STEVOR are bordered with red lines in Figure 5). Similar to the STEVOR, RIFINs cluster into one main clade which includes the majority of the multigene family and a second, smaller clade which shows a higher divergence from the rest. The divergent groups from both multigene families however appear to be closely related to each other, highlighting the similarities between the two families and suggesting the possibility of a common ancestor. From an evolutionary perspective it appears that a small subset of *rif* sits between the *stevor* and *rif* multigene families, further strengthening the observations described (Otto et al., 2018). Taken into account the heterogeneity within the STEVOR multigene family, especially within clade B, we decided to expand the search for potential interaction partners to cover the entire diversity of this multigene family.

Figure 5: Alignment of the amino acid sequence of all small variant surface antigens

Alignment of all small variant surface antigens groups STEVOR and RIFIN into two distinct, separate groups. Red lines indicate STEVOR. Both multigene families show a core group of genes with minimal diversity and a small subset of genes with increased divergence. The divergent groups appear to be intermediates between the two multigene families, suggesting a common ancestor for both.

3.1.3. Screen of 9 STEVOR for red blood cell binding

The identification of STEVORs that interact with glycophorin C was based on expression of the semiconserved domains of three different STEVOR on the surface of green monkey kidney (COS7) cells (Niang et al., 2014). The proteins were cloned into pDisplay plasmids for surface expression on mammalian cells. The plasmid adds an N-terminal Ig κ -chain leader sequence to the protein of interest

which causes export of the protein onto the surface of the cell. Furthermore, it provides a platelet derived growth factor receptor transmembrane domain to anchor the fusion protein onto the surface of the target cell. To detect the protein on the surface of the target cell, the plasmid adds a hemagglutinin and c-myc tag to the protein (at the N- and C-terminus, respectively). In order to expand the study to cover further STEVOR, the semiconserved region of 9 additional STEVOR (7 Clade A and 2 Clade B) were cloned into the pDisplay plasmid. COS7 Cells were transfected using Lipofectamine 2000. Transfection efficacy was assessed by live cell staining with α c-myc antibody followed by detection using fluorescently labelled secondary antibodies. Surface expression of the fusion protein was only detected in about 1% of the population. To assess the ability of STEVOR to form rosettes, the cells were incubated with human red blood cells. After washing away the unbound red blood cells, COS7 cells were observed under a light microscope using a 20x objective and cells with at least 3 bound red blood cells were counted as rosette (Figure 6a). For each construct, at least 1000 cells were counted. The percentage of rosette forming cells was calculating assuming 1% transfection efficacy.

Figure 6: Expression of STEVOR semiconserved region on the surface of COS7 cells

The semiconserved regions of 9 different STEVOR were cloned into the pDisplay plasmid for surface expression on mammalian cells. Green monkey kidney cells (COS7) were transfected with the constructs and assessed for their ability to form rosettes. (a) COS7 cells expressing the semiconserved region of Pf3D7_0102100 bind

uninfected red blood cells. Transfected cells were incubated with red blood cells. After washing away of unbound cells, COS7 cells binding at least 3 red blood cells were counted as rosette. Dead, rounded cells (right side) were regularly observed to form exceptionally big rosettes. (b) Screen of 7 Clade A and 2 Clade B (indicated with the letter in brackets) for their ability to form rosettes. Transfection efficacy was shown to be 1% in an independent experiment by detection of the c-myc tag on the surface of live cells after transfection. For each assay, at least 1000 COS7 cells were counted, with or without formed rosette. Out of all counted cells, 1% was considered “transfected”. Finally, rosette forming cells were compared to “transfected” cells and percentage was calculated. All tested STEVOR constructs formed rosettes, albeit with varying efficacy. Pf3D7_0102100 served as positive control.

The previously described Pf3D7_0102100 was used as a positive control. Even though all of the 9 STEVOR appear to be able to form rosettes, their efficacy to do so varies (Figure 6b). While the strongest construct (Pf3D7_0425500) shows rosettes in about 13% of all transfected cells, the weakest one does so only in 2%. Considering the low transfection efficacy of about 1%, this resulted in very few rosettes per assay. Furthermore, the rosetting frequency of Pf3D7_0102100 was consistently below rate described previously by Niang et al. This could potentially be due to uncertainty about the passage number of the COS7 cells used. With increasing passage number, COS7 cells expose less protein on their surface which would result in fewer rosettes formed. Another observation was that cells that appeared to be dying (rounded cell shape, Figure 6a right cell) caused formation of rosettes with more red blood cells than healthy ones (spread out cell, Figure 6a left cell). Considering that no toxic effects of STEVOR expression have been described before, this could be another indication of high passage number. Finally, since this assay relies on constant re-transfection of cells in combination with re-evaluation of the transfection efficacy, an alternate approach to study STEVOR – ligand binding was attempted.

3.1.4. Recombinantly expressed STEVOR semi-conserved region binds to unidentified red blood cell ligand

Instead of expressing the semiconserved region of STEVOR on the surface of mammalian cells, binding to red blood cells was to be tested using recombinant protein. The following experiments were performed by Chua Zong Sheng under the supervision of the author. The semi-conserved regions of three STEVOR (Pf3D7_0222800, Pf3D7_0832600 and Pf3D7_1400700, Clade A, B and A respectively) were cloned into pET24a plasmids for bacterial expression. The same sequences that were used for the previously described mammalian expression were used for bacterial expression. The pET24a plasmid adds a C-terminal 6 histidine tag for purification purposes and protein detection. An expression system, Rosetta(DE3) cells were chosen. This strain carries a T7 polymerase under *lacUV5* promoter control, enabling IPTG dependant protein expression. Furthermore, it harbours additional tRNAs for better translation efficacy of eukaryotic proteins.

Expression was induced with IPTG (Isopropyl- β -D-thiogalactopyranosid) (Pf3D7_0222800 and Pf3D7_1400700: 200 μ M, Pf3D7_0832600: 400 μ M) at an OD₆₀₀ of 0.2 for 6 hours at 30°C while shaking. Bacteria were lysed by sonication, followed by centrifugation to remove insoluble proteins. The cleared lysates were incubated with Ni-NTA beads. Beads were subsequently washed two times and bound protein was eluted with imidazole. Since contaminating bacterial proteins might interfere with protein binding, purity of the eluted protein was determined by Coomassie staining. To this end, fractions of input, flowthrough, washes and elutions were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. (Figure 7, Pf3D7_0832600 not shown).

Figure 7: Purification of the semiconserved region of two different STEVOR from bacterial lysates

Purification of the semi-conserved regions of Pf3D7_0222800 and Pf3D7_1400700 from *E. coli* cells. The protein sequence was cloned into pET24a plasmids, adding a C-terminal 6xhistidine tag to the protein. Plasmids were transformed into Rosetta(DE3) cells. Cells were lysed using sonication and lysates were cleared by centrifugation. Protein was captured from the soluble fraction using Ni-NTA beads and eluted using imidazole. Image of Pf3D7_0831600 has not been recorded and is missing in this figure.

To validate functionality of the recombinant protein, its ability to bind to receptors on the surface of red blood cells was tested. To reduce complexity of the red blood cell surface receptor repertoire, cells were either shaved using trypsin or chymotrypsin or treated with neuraminidase prior to binding (Duraisingh, Maier, Triglia, & Cowman, 2003). While trypsin preferentially cleaves after lysine and arginine residues, chymotrypsin cleaves at hydrophobic amino acids (tyrosine, tryptophan, and phenylalanine). This results in cleavage of different subsets of proteins. Neuraminidase on the other

hand cleaves glycosidic linkages of neuraminic acids. Sialic acid dependant interactions are therefore susceptible to neuraminidase treatment. Limiting the availability of surface receptors has been used to identify invasion related receptors previously. Trypsin treatment ablates the ability of parasites to efficiently invade red blood cells due to the cleavage of Glycophorin A (Hadley et al., 1987). Glycophorin B on the other hand is sensitive to chymotrypsin but can resist tryptic digest (Thompson, Triglia, Reed, & Cowman, 2001). Depending on the invasion pathway utilized by a particular parasite strain, these treatments might limit their ability to invade red blood cells. Glycophorin C is sensitive to tryptic digest but resists chymotrypsin. Furthermore, binding of STEVOR to Glycophorin C is sialic acid dependant. Recombinant STEVOR protein interacting with Glycophorin C would therefore show reduced binding to red blood cells pre-treated with trypsin or neuraminidase as compared to untreated control, while treatment with chymotrypsin would have no significant effect. This pre-treatment of red blood cells not only served as control that binding of the recombinant protein is specific, it also indicates interaction with novel ligands.

Elution 1 of the three proteins was incubated with red blood cells (untreated, trypsin shaved, chymotrypsin shaved and neuraminidase treated) in RPMI, supplemented with 10% fetal bovine serum. Unbound protein was separated from the cells by centrifugation with dibutyl phthalate (DBP). In short, the cell suspension is overlaid with DBP. After centrifugation, the red blood cells will pellet on the bottom of the tube, separated from the protein solution by the layer of DBP. Finally, the bound protein is eluted with high salt and detected by western blot (Figure 8).

Two of the proteins (Pf3D7_0222800 and Pf3D7_1400700, both Clade A) show binding to untreated and chymotrypsin treated cells, but not to trypsin or neuraminidase treated cells. The protein Pf3D7_0222800 however shows bands both above as well as below the expected size. The band below at about 11kDa could possibly be a degradation. It is of note that the band corresponding to a short fragment shows a higher intensity than the correct fragment. Furthermore, two bands of higher molecular weight can be detected at about 30 and 32kDa. Those bands were not detectable on the Coomassie stained gel in Figure 7. It is unknown where what these bands could correspond to, but

dimerization of RIFIN has been reported before (Goel et al., 2015). This binding pattern would be expected from glycophorin C interacting STEVORs. Pf3D7_0832600, the Clade B STEVOR, did only bind untreated red blood cells. All three pre-treatment conditions disrupted binding below detectable levels. This suggests that Pf3D7_0832600 interacts with a novel, so far unidentified receptor on the surface of red blood cells.

Figure 8: Binding of the semiconserved region of three different STEVOR to red blood cells

The recombinantly expressed semi-conserved region of three different STEVOR was incubated with red blood cells. Prior to binding, red blood cells were either kept untreated, shaved with either trypsin or chymotrypsin or treated with neuraminidase to reduce surface receptor complexity. Bound protein was eluted in high salt and detected using western blotting. Red arrows indicate protein of interest. Pf3D7_0222800 and Pf3D7_1400700

binding is sensitive to trypsin and neuraminidase treatment but unaffected by chymotrypsin digest, suggesting interaction with glycophorin C. The Clade B STEVOR Pf3D7_0831600 shows an unexpected susceptibility to all three types of treatment.

Despite the success of recombinant protein in identification of receptor binding, the methodology is cumbersome and inaccurate (Goelet al., 2015). Each fragment requires optimization of expression and purification protocols. Correct folding and function are difficult to control, as a lack of binding to red blood cells could result either from incorrect folding as well as binding of the protein to a receptor which is not present on red blood cells. Furthermore, bacteria lack certain post-translational modifications which might be necessary for correct ligand interaction. Utilizing recombinant protein is therefore more suitable as a secondary approach to validate protein binding identified through different means. The most successful method has been phenotypic enrichment of parasites, which is time-consuming and restricted to proteins that are enriched by chance. It was therefore necessary to develop a new platform to study multigene families expressed by the parasite itself.

3.2. Establishment of a platform to study VSA

Considering the essential role that multigene family members play in parasite survival *in vivo* and the tremendous effort invested by the parasite into their correct expression, information on their function is relatively scarce. Only a small percentage of proteins per multigene family has been studied. This is largely due to the fact that to date only a limited number of approaches have been available to researchers to study a single defined member of a multigene family in detail: single cell cloning, phenotypic enrichment and overexpression. Single cell cloning results in populations of cells with random multigene expression patterns, which require extensive profiling to verify and might not yield any suitable parasites after all (Neveu et al., 2018). Phenotypic enrichment is laborious and restricted to proteins with a phenotype that enables selection to begin with (Scherf et al., 1998; Schieck, Pfahler, Sanchez, & Lanzer, 2007). Overexpression struggles with the size of PfEMP1 and can cause misleading results considering the lack of temporal control due to the use of constitutively active promoters (Horrocks & Lanzer, 1999). Furthermore, parasites switch their multigene family expression pattern in culture, thus requiring constant verification and reselection (Blythe et al., 2008; Horrocks, Pinches, Christodoulou, Kyes, & Newbold, 2004).

The study of multigene families is further challenged by the difficulty in the generation of specific antibodies against a unique member due to their high sequence similarity. Finally, the study of epigenetic control within and between multigene families relies on episomal expression of promoter regions (Araujo et al., 2018; Dzikowski et al., 2006; Voss et al., 2006). This approach however removes these control elements from their genomic locus and might bypass currently unidentified mechanisms of epigenetic crosstalk. This has meant that despite the importance of studying multigene families the lack of suitable experimental tools has hampered progress in the past decades.

This chapter aims to address this challenge and describes a novel approach to study VSA in *P. falciparum*. To do so, the novel endogenous tagging method- the 'selection linked integration' (SLI) was adapted to force the parasite to express a *stevor* of choice under the control of its endogenous promoter (Figure 9) (Birnbaum et al., 2017). SLI takes advantage of a ribosomal skip peptide, which

causes co-translational break of the amino acid strand, to generate 2 proteins from a single mRNA molecule (Donnelly et al., 2001). Endogenous tagging of any gene of interest with a skip peptide followed by a drug resistance marker enables positive selection for genomic integration since the resistance marker is only expressed after integration. If this approach is used on multigene family members, simultaneous selection for parasites expressing this particular protein is achieved.

Figure 9: Schematic of Selection Linked Integration

The plasmid, which lacks a *Plasmodium* promoter, includes a single recombination arm (without a stop codon), followed by a tag of choice, the T2A skip peptide and a Neomycin resistance marker. Due to the high sequence homology of multigene families, the recombination arm includes the 750 base-pairs at the C-terminus of the gene of interest. Recombination can happen at any point within the single recombination arm and causes integration of the entire plasmid. After integration, mRNA spanning the entire gene, the tag and the neomycin resistance marker will be transcribed. The skip peptide causes break of the amino acid strand during translation, resulting in two independent proteins. Correct integration is validated by PCR (Primer localisation is indicated).

3.2.1. Validation of SLI integration into three different *stevor* loci

To validate whether the SLI method can be used to force expression of members of multigene families, tagging of three different *stevors* in the standard laboratory strain 3D7 was attempted. All three different genes (Pf3D7_0102100, Pf3D7_0631900 and Pf3D7_1149900) are part of Clade A. One of the proteins, Pf3D7_1149900, has not been studied in depth before. The second protein, Pf3D7_0631900, has been described in two previous publications (Naissant et al., 2016; Wichers et al., 2019). It appears

to be transported, potentially through Maurer's Clefts, to the surface of infected red blood cells in asexual parasites. In sexual stages, it is interacting with the spectrin cytoskeleton of the infected red blood cell. The third protein, Pf3D7_0102100, has been shown to interact with glycophorin C on the surface of infected red blood cells (Niang et al., 2014) and was used as positive control in the previously described COS7 cell binding assay. A triple hemagglutinin (3HA) tag was added to detect the fusion protein. The resulting cell lines were termed 0102100.3HA, 0631900.3HA and 1149900.3HA, depending on the *stevor* targeted.

One of the difficulties of working with multigene families is the high sequence similarity in certain regions of the protein. The SLI method utilizes single recombination arm cross-over to integrate into the genome at its target site, specifically at the 3' end of the gene of interest. All three variant surface families however show the highest degree of similarity exactly at these regions, making primer design problematic. Specifically, primers at the very 3' end of the gene show perfect matches for several different genes. Amplification of the recombination arm from genomic DNA followed by cloning into the plasmid regularly resulted in the generation of several different constructs. Sequencing confirmed that these constructs include correct recombination arms for several different *stevor*. Fortunately, the 5' end of the recombination arm reaches into the less conserved parts of the protein, causing integration into the correct locus.

Integration was validated by PCR (Figure 10). The same set of three PCR reactions that was used in the original SLI study was used to verify correct genomic integration and expected band sizes are indicated in Figure 10 (Birnbaum et al., 2017). The first reaction, termed 5', starts within the gene of interest just 5' of the recombination arm and ends within the linker and before the tag (primers G_fwd and P_rev are indicated in Figure 9). The second reaction, termed 3', starts just 5' of the recombination arm on the plasmid and ends 3' of the gene of interest (primers P_fwd and G_rev are indicated in Figure 9). During integration, the double strand break can happen at any point within the single recombination arm. The remainder of the plasmid, including the parts of the recombination arm that are located 5' of the double strand break, is integrated downstream of the gene of interest. This brings the primers

P_fwd and G_rev into close proximity. Considering that the exact location of the double strand break cannot be predicted, only an estimate of the 3' PCR product can be given. The final reaction uses the two primers located on the genome and spans the unmodified locus. Upon successful integration, the transfected cell line should give bands in the 5' and 3' reaction but not for the genomic locus. Genomic DNA of the parental 3D7 cell line on the other hand should only give a band for the genomic locus.

Figure 10: Validation of genomic integration by PCR

PCRs spanning the 5', 3' and genomic loci of the three targeted stevor Pf3D7_0102100 (a), Pf3D7_0631900 (b) and Pf3D7_1149900 (c) in the transfected cell lines and the parental 3D7. Presence of bands in the 5' and 3' reactions validate correct integration of the plasmid into the targeted locus. Loss of the genomic locus proves complete integration in the entire population without wild type escape. Expected band sizes are given in the

table. Integration into the Pf3D7_0102100 only yielded positive PCR for the 5' arm, and the genomic locus was retained. Integration into Pf3D7_0631900 showed bands of the expected size in both 5' and 3' arm and loss of the genomic locus. Integration into Pf3D7_1149900 was positive for 5' and 3' PCR, but retained the genomic locus. (d) Table indicating the predicted sizes of the PCR products. Length of 3' arm can only be given as approximation as exact position of integration is unknown.

Integration was validated to varying degrees between the three cell lines. Only one PCR validating 0102100.3HA integration, the one spanning the 5' arm, gave a band of the expected 618bp size. The cell line retained the genomic locus and was negative for the 3' arm. To verify that the plasmid was integrated into the correct locus, the PCR spanning the 5' arm was sequenced, confirming correct integration. Validation of 0631900.3HA on the other hand gave PCR products of the expected size of 888bp for the 5' and about 900 bp for the 3' arm, as well as loss of the genomic locus. PCR of 1149900.3HA showed the expected sizes of 866 and about 900 bps for the 5' and 3' arm, respectively. Although a PCR product for the unmodified locus was still detected this most likely was due to amplification of another *stevor* locus. Homology within the multigene family is highest at the 3' end, since the C-terminal region of the protein is highly conserved. Due to this homology, primers within this region tend to be less specific which could lead to amplification of another, similar locus. In order to verify that the plasmid integrated into the correct locus, the PCR spanning the 5' arm was purified and sequenced, confirming the integration. It is possible to exclude persistence of the endogenous locus through a variety of methods. Whole genome sequencing or southern blot analysis could identify wild type escape within the population. Purification of the "locus" PCR followed by sequencing would similarly tell whether the entire population is homogenous.

3.2.2. All three targeted STEVOR are exported into the host cell

Correct expression of the fusion protein and complete skip peptide cleavage was validated by western blot analysis (Figure 11). Late stage parasites were enriched on a percoll gradient. Parasites were extracted from the host red blood cell by lysis with 0.05% saponin. Saponin is a plant derived detergent which specifically attaches to cholesterol and therefore preferentially lyses the high cholesterol red

blood cell membrane over the low cholesterol parasite membrane (Ansorge et al., 1996; Ansorge, Paprotka, Bhakdi, & Lingelbach, 1997). By separation of the infected red blood cell into the purified parasite (termed Parasite) and the supernatant containing the red blood cell membrane, cytosol and membranous compartments (termed Host), export of the protein beyond the parasite boundaries was assessed. The freed parasites were lysed in RIPA buffer to achieve complete solubilization. Plasmodium aldolase, a parasite internal protein, served as loading as well as fractionation control (Knapp, Hundt, & Kupper, 1990).

Figure 11: Western blot analysis of STEVR knock-in cell lines

(a) Late stage parasites were enriched on a percoll gradient and extracted from the host red blood cell by saponin lysis, resulting in “Parasite” fractions of the purified parasite and “Host” fractions including the red blood cell membrane, membranous compartments within the host and its cytosol. Plasmodium aldolase, a parasite internal protein, served as loading control. Bands are highlighted for 1149900.3HA. All three tagged STEVR are present in both fractions, suggesting export of the protein beyond the red blood cell membrane. Surprisingly, 0102100.3HA is only about half the expected size. (b) Table indicating predicted protein weight in kDa. (c)

All three proteins are expressed and present both in the parasite as well as the host fraction, suggesting export of the protein beyond the boundaries of the parasite. Strikingly, while 0631900.3HA and 1149900.3HA are expressed at the expected size of 40.9 or 41.7kDa respectively, 0102100.3HA showed a size of only about 20kDa. Considering that the 3HA-tag with linker sequences has a molecular weight of 6.8kDa, the tagged STEVOR seems to be truncated to a fragment of approximately 13kDa. This particular gene is annotated as a pseudogene on Plasmo_DB and is lacking an ATG codon at the expected position according to the 3D7 reference genome.

To unravel whether this gene actually lacks the correct start codon and is therefore forced to utilize an alternate one, is differentially spliced or truncated at a post-translational level, amplification of the gene from cDNA was attempted using a fixed reverse primer and two different forward primers, one of which at the start of the gene ("Full length") and one which sits in the centre of the gene ("Short") (Figure 11c). Only the central primer lead to amplification of a DNA fragment, suggesting that the observed truncation happens already on a transcriptional level.

3.2.3. STEVOR Pf3D7_0631900 is exported onto the surface of the infected red blood cell

In order to test for surface exposure of the tagged STEVOR proteins, we performed surface trypsin treatment of live trophozoites. Since the 3HA tag remains within the host red blood cell, surface shaving of infected red blood cells would result in a truncated form of the protein. Complete shaving was controlled by probing for Glycophorin C. Host cell integrity is controlled by normalization to a red blood cell cytosolic protein. Since all surface exposed trypsin sensitive proteins are cleaved, the reduction in signal intensity of full length STEVOR corresponds to intracellular protein which remains protected from tryptic digest, allowing us to estimate the percentage of exported protein. Furthermore, the molecular weight of the cleaved product allowed us to estimate the membrane topology of this STEVOR. Late stage parasites were enriched on a percoll gradient and treated with 0.5mg/ml trypsin for 30 minutes. The reaction was stopped with 1mM Phenylmethylsulfonyl fluoride (PMSF). Proteins

were solubilized in RIPA buffer and analysed by western blot (Figure 12a). In order to quantify the amount of exposed protein, the signal of the full length protein is normalized to GBP130, a soluble parasite protein which is being exported to the host cell cytoplasm (Nolte, Hundt, Langsley, & Knapp, 1991). Glycophorin C served as positive control for complete shaving of the red blood cell surface. Even though the predicted mass of glycophorin C is about 13.8kDa, extensive glycosylation of the protein shifts the detected weight of the protein consistently to about 35kDa (Furthmayr, 1978; Smythe, Gardner, & Anstee, 1994). Only one of the three STEVOR, Pf3D7_0631900, showed significant cleavage of approximately 22% of total protein, confirming surface exposure of the protein (Figure 12b). This finding suggests that not all STEVOR are trafficked to the surface of the infected red blood cell but that some of them remain within the parasite. This is in agreement with previous studies that highlight diversity in localization within the STEVOR multigene family and resistance of some STEVOR to trypsin treatment (Bachmann et al., 2015). It also indicates that even among the STEVOR that are displayed on the red blood cell, only a minor part of the total protein is transported all the way onto the surface while a majority remains within the cell. It has been previously described that PfEMP1 remains largely within the infected red blood cell as well, suggesting that this is a common characteristic of surface antigens (Waterkeyn et al., 2000).

Figure 12: Western blot analysis of trypsin shaved parasites shows that Pf3D7_0631900 is exported onto the surface of the infected red blood cell

Late stage parasites expressing specific STEVOR were enriched on a percoll gradient and shaved using 0.5mg/ml trypsin. Reaction was stopped with PMSF and cells were lysed with RIPA buffer. Lysates were analysed by western blot. (a) Western blot analysis of parasite lysates after trypsin shaving (+Trypsin) or without treatment (-Trypsin). Glycophorin C was used as positive control and confirmed complete shaving of red blood cell surface (red arrow indicates cleavage product). Green arrows indicate Pf3D7_0631900.3HA, red arrows indicate glycophorin C full length as well as cleaved. Only Pf3D7_0631900.3HA shows a smaller band after trypsin treatment. (b) Quantification of band intensity of the full-length protein with or without trypsin treatment. Signal intensity is normalized to GBP130, a soluble parasite protein in the host cell cytosol. Signal of untreated cells is set to 100%. Signal of Pf3D7_0631900.3HA is significantly reduced by 22% after trypsin treatment, confirming surface exposure of the protein. ** Indicates significant reduction in signal intensity (2 sample T-test: $p=0.00807$)

3.2.4. Pf3D7_0631900 is anchored in the host membrane at its central transmembrane domain

The post-cleavage fragment of Pf3D7_0631900.3HA is about 22kDa in size (Figure 12a). STEVOR proteins have generally two predicted transmembrane domains, one central of the protein and one at its C-terminus (Cheng et al., 1998). The membrane topology of STEVOR are part of an ongoing debate.

One previous study suggested that STEVOR are inserted into the host cell membrane with their central transmembrane domain (Bachmann et al., 2015). Two recent publications however propose insertion in the C-terminal domain or a two transmembrane domain model where the C-terminal domain and the very N-terminal signal peptide are integrated into the membrane (Andersson et al., 2019; Wichers et al., 2019). The central transmembrane domain is predicted to span amino acids 175 – 197 (Figure 13a) according to PlasmoDB 9.0 (Aurrecochea et al., 2009). Prediction of tryptic cleavage by the ExPASy PeptideCutter tool shows a stretch of several potential sites between the amino acid positions 157 – 177 (Figure 13b) (Wilkins et al., 1999). A total of 21 cleavage sites can be found throughout the protein. Furthermore, another stretch of cleavage sites covers the C-terminal domain of the protein at amino acids 284 – 299 (7 sites) (not shown). Cleavage at position 157 would result in a product of 22.7kDa, coinciding with the observed size. The molecular weight of the cleavage product obtained after tryptic digest therefore suggests that this particular STEVOR is anchored into the host plasma membrane through its central transmembrane domain. Early studies suggested a two transmembrane topology, whereby both the N- and C- terminal ends of the protein remain of the same side of a membrane (Cheng et al., 1998). Considering that the C-terminal tail of the STEVOR (including the 3HA tag) remains detectable while glycophorin C is entirely cleaved from the surface of the red blood cell, a two transmembrane topology appears unlikely for Pf3D7_0631900. Both recent studies suggesting different membrane topologies utilize expression systems other than *P. falciparum*. Both recent studies utilize the TNT quick coupled transcription/translation kit with microsome-supplementation (Andersson et al., 2019; Wichers et al., 2019). Even though the expression system is capable of efficient protein glycosylation, it might not reflect the situation observed within the parasite. Furthermore, the study suggesting a two-transmembrane topology included the signal peptide into their constructs. Considering that the signal peptide is cleaved during protein export in *P. falciparum*, the experimental setup resembles the situation within the endoplasmic reticulum rather than in the host red blood cell (Boddey et al., 2013; Boddey et al., 2010). Considering that the findings presented here are based on STEVOR in their native environment, expressed by the parasite itself instead of an artificial expression

system, it is likely that this experimental approach resembles the true membrane insertion most closely. Furthermore, these findings coincide with a previous study utilizing the same approach (Bachmann et al., 2015).

Figure 13: Several tryptic cleavage sites are predicted N-terminal of the central transmembrane domain of Pf3D7_0631900

(a) Structural prediction of Pf3D7_0631900 shows presence of two transmembrane domains. The central domain spans amino acids 175 – 197 while the C-terminal one spans amino acids 264 – 286. (b) Nine tryptic cleavage sites are predicted to be N-terminal of the central transmembrane domain, spanning amino acids 157 – 177. A similar stretch of cleavage sites is detected at the amino acids 284 – 299 as well as scattered sites throughout the protein (not shown).

3.2.5. Immunofluorescence analysis of the three STEVOR knock-in cell lines confirm export beyond the parasite boundaries

We further validated protein localization by IFA on early trophozoite stage parasites as well as merozoites (Figure 14). All three STEVOR showed a distinctly different localization, both in trophozoite stage as well as merozoites. Trophozoites were fixed using methanol while merozoites were fixed in 4% paraformaldehyde (PFA) followed by membrane permeabilization in 0.1% saponin. To confirm export of the protein beyond the boundaries of the parasite, cells were probed for the 3HA tag and EXP2, a protein residing in the parasitophorous vacuolar membrane (PVM)(Johnson et al., 1994). The

small exported membrane protein sEMP1, a Maurer's Cleft resident protein, served as marker for the Clefts (Dietz et al., 2014). Finally, merozoites boundaries were stained with Gap45. The protein is an essential part of the glideosome, the motor complex engaged in invasion of merozoite (Baum et al., 2006). DNA was stained with Hoechst33342.

Pf3D7_0102100.3HA, the "short" STEVOR, seemed to largely remain within the parasite (Figure 14a). A majority of the protein signal overlaps with EXP2, indicating incomplete export. The exported part of the protein could be seen as spots throughout the host cell. The spots show a partial overlap with sEMP1 (green arrows), indicating that the protein is trafficked to the Maurer's Clefts. The protein can be found all throughout the merozoite.

Figure 14: Localization of STEVOR in asexual stages by immunofluorescence assay

Parasites were fixed using methanol (trophozoite) or PFA (merozoite). Protein localization was determined by

co-localization with EXP2 (PVM), sEMP1 (Maurer's Cleft) and Gap45 (glideosome). DNA was stained with Hoechst3334s. (a) Pf3D7_0102100.3HA remains to large parts within the parasite. The exported protein co-localizes with Maurer's Clefts. It is present all throughout the merozoite. (b) Pf3D7_0631900.3HA is exported into the red blood cell where it forms a ring at the periphery, indicative of red blood cell membrane association. It shows partial overlap with Maurer's Clefts and localizes to the apical tip of merozoites. (c) Pf3D7_1149900.3HA is largely localizes to spots within the host red blood cell. These spots overlap with Maurer's Clefts. The protein is absent from merozoites.

The surface exposed STEVOR Pf3D7_0631900.3HA could be detected all throughout the infected red blood cell and showed a distinct ring around the cell, characteristic of proteins associated with the red blood cell membrane (Figure 14b). Compared to Pf3D7_0102100.3HA, the majority of protein appears to be exported beyond the EXP2 signal. The cell population showed a certain degree of variability with some cells displaying a "smooth" staining of the infected red blood cell (seen in the co-staining with EXP2) while others appeared more as spots (seen in the co-staining with sEMP1). Those spots partially co-localized with sEMP1 (highlighted with green arrow), indicating export through the Maurer's Clefts. In contrast to Pf3D7_0102100, the protein could be found as a single, distinct punctate in each cell. STEVOR localizing to the apical tip of merozoites have been associated with merozoite invasion (Khattab et al., 2008). A recent publication investigated localization of this specific STEVOR using specific antibodies raised against it (Wichers et al., 2019). The localization observed is highly similar to the one described here. This strengthens the assumption that tagging a STEVOR using the SLI method does not alter protein trafficking and export but retains its native localization.

The third STEVOR, Pf3D7_1149900.3HA, appeared to be entirely exported beyond the parasite boundaries (Figure 14c). It remained in punctates which localized with sEMP1 (green arrow). After schizont ruptures, those punctates remained as a "cloud" surrounding the merozoites. There is no evidence that this STEVOR is expressed in the merozoite.

In summary, each of the three targeted STEVOR displays a unique localization both in trophozoites as well as merozoites. Since the large phenotypic diversity within the STEVOR multigene family has been

described before, it can be assumed that the knock-in method has no effect on protein localization (Bachmann et al., 2015; Wichers et al., 2019). Furthermore, one of the *stevor* is not detected in merozoites despite being targeted by the SLI method. Differential peak expression among different *stevor* has been reported recently. Both Pf3D7_0631900 as well as Pf3D7_1149900 show a peak expression at early trophozoite stage and no expression at later stages. This suggests re-localization of already translated Pf3D7_0631900 into the free merozoites while Pf3D7_1149900 remains at its original localization. It was unclear whether targeting of a gene with the SLI method would cause changes in temporal expression due to the persistence of drug pressure throughout the entire life-cycle. The absence of Pf3D7_1149900 from merozoites however suggests that gene knock-in does not introduce another, second peak of expression, confirming that the SLI method has an effect on the temporal expression pattern of the targeted gene.

3.2.6. STEVOR are expressed in gametocytes but remain within the parasite

STEVORs have been previously described to be exported in gametocytes and exposed on the red blood cell surface (Khattab et al., 2008; McRobert et al., 2004). Furthermore, cleavage of the protein appears to be essential for stage IV – V transition (Tiburcio et al., 2012). Export of the three targeted STEVOR was therefore assessed by IFA on stage IV gametocytes (Figure 15). In contrast to the asexual stages, all STEVORs showed the same localisation (even though the expression levels appeared to vary). The protein is only present within the parasite, where it displays a granular pattern throughout the cell. This pattern remains constant throughout gametocyte development (data only shown for stage IV gametocytes).

It is unclear why STEVOR that are exported in asexual stages remain within the parasite in gametes. Since all three proteins are exported in asexual stages, adverse effects of the tag to protein export can most likely be excluded. It has been described before that B-type RIFINs are not exported during gamete stages. It is conceivable STEVOR behave similarly, with only a subset of them being exported onto the surface of gametocytes.

Figure 15: Localization of STEVOR in gametocytes by immunofluorescence assay

Stage IV gametocytes of the three STEVOR knock-in cell lines were fixed with methanol and probed for EXP2 and the targeted STEVOR. DNA was stained with Hoechst33342. All three of the proteins are expressed in gametocytes where it remains parasite internal.

3.2.7. Knock-in of stevor retains their localization and temporal expression

The three different STEVOR studied underly the so far unexplored diversity within this family with each protein displaying a unique phenotype. While all three proteins are exported into the host red blood and show partial co-localization with sEMP1, suggesting localization to the Maurer's Clefts, none of them is exported in gametocytes. This is in line with unsuccessful recent efforts to identify STEVOR that are presented on the surface of sexual stage parasites (Neveu et al., 2018). Out of the three, only Pf3D7_0631900 is transported to the red blood cell membrane and finally presented on the surface of the infected cell. According to the approximate size of the cleavage product, this STEVOR is anchored into the host red blood cell membrane using the central predicted transmembrane domain. This finding contradicts previous reports which suggest usage of the C-terminal transmembrane domain (Bachmann et al., 2015). Considering the variety between STEVOR observed in this work, different

STEVOR might utilize either if the two domains or even both. Depending on the topology, the predicted function of the protein domains might differ as well. The hypervariable region, which is predicted to be between the two transmembrane domains, supposedly facilitates antigenic variation. In STEVORs that utilize the central transmembrane domain however the hypervariable region would be within the host cell, potentially playing a role in so far undescribed processes. The intracellular domain is being phosphorylated by protein kinase A, indicating functions beyond anchorage of the protein (Naissant et al., 2016). The second *stevor*, Pf3D7_1149900, appears to remain within the Maurer's Clefts during the entire life cycle. This is highlighted by the observation that it remains as a cloud of protein surrounding merozoites after schizont rupture. It is the only protein described in this work among all three multigene families that is not present in merozoites. The final *stevor* targeted is Pf3D7_0102100, the strongest glycophorin C binder described previously (Niang et al., 2014). Binding of the STEVOR to glycophorin C was shown by expression of the protein on the surface of COS7 cells. The same construct served as positive control in the COS7 cell binding assay performed in this work. The one STEVOR that was episomally expressed in *P. falciparum* was "ST1", PF3D7_1040200. Surprisingly, forcing the parasite to express Pf3D7_0102100 resulted in a protein which was only about 20kDa in size, much smaller than the predicted weight. Since it is not exported onto the host cell surface, its ability to cause rosettes could not be evaluated. According to Plasmo_db this protein is lacking an ATG codon at the predicted site and is therefore considered a pseudogene. Consistently with this, the mRNA does not cover the entire predicted coding sequence but only the second half, suggesting the shortening of the protein is indeed due to usage of an alternative start site and not alternative splicing. Since glycophorin C binding has been shown for the N-terminal, semi-conserved domain, which is apparently missing in this cell line, we predict that the protein would be unable to bind glycophorin C even if it would be presented on the host cell, at least in the 3D7 lab strain. Genes with high sequence similarity (>98%) to Pf3D7_0102100 can be found in several in other strains of *P. falciparum* such as IT, HB3, KH1, KH2 and GB4, originating both from Africa (GB4, Ghana) as well as South America (HB3, Honduras). This similarity in itself is striking considering the high diversity observed in *var* genes surveyed across

different, geographically proximal sites (Day et al., 2017; Ruybal-Pesantez et al., 2017). It would be interesting to see whether all strains display the same truncation. The strains GB4 as well as HB3 both share 99.76% sequence similarity and both lack the start codon, analogous to 3D7.

One consideration of the method applied in this work was whether it would alter the temporal expression pattern or cause upregulation of the targeted genes. This can be excluded due to several observations. First, expression of the targeted *var* is strongest at 18 hours post invasion (among the timepoints investigated in this work) and is reduced below detectable levels shortly after, an expression pattern described before for this multigene family (S. Kyes et al., 2000). Active expression of the targeted locus throughout the entire intra-erythrocytic life cycle is therefore not necessary to maintain resistance to the drug. Second, relative transcript levels differ between the multigene families, confirming that even low abundance of the resistance conferring protein is sufficient and upregulation of the gene is not necessary (Birnbaum et al., 2017). Finally, one of the STEVOR is absent from merozoites, further confirming that stage specific expression is maintained. Considering that the targeted proteins display highly diverse localizations while maintaining their described functionality shows that the tags described in this work do not alter protein export and correct localization. A recent study suggested different expression peaks among different *stevor* (Wichers et al., 2019). The two genes Pf3D7_0631900 as well as Pf3D7_1149900 peak at early trophozoite while Pf3D7_0102100 peaks at late schizont stage. A future experiment might study expression of the targeted locus throughout the 48hour life cycle and study expression peaks to confirm the published results. However, all these observations combined prove that the methodology described in this work is robust and can be applied to study any member of multigene families in their endogenous environment.

3.2.8. Expanding the knock-in method to the *var* family

The previous chapters discussed how three members of the *stevor* multigene family were targeted with the SLI method and successfully knocked-in. In order to show that the method can be applied to all major VSA families in *P. falciparum*, it has to be expanded to the *var* and *rif* as well. The next

chapter discusses the effort to target the so far uncharacterized *var* Pf3D7_0421300 with SLI and the functional validation of the resulting fusion protein.

3.2.9. Validation of SLI integration into the targeted *var* locus

In order to tag Pf3D7_0421300, a 743 base-pair recombination arm was cloned between the NotI and MluI sites of pSLI, resulting in the excision of the 2xFKBP domains present in the original plasmid. This resulted in tagging of the PfEMP1 only with GFP. Designing primers to amplify the recombination arm suffered from the same problems described in chapter 3.2.1.

Integration was confirmed by PCR using gDNA of the transfected cell line and parental 3D7 (Figure 16). Primer pairs were chosen as discussed in chapter 3.2.1. In short, the 5' arm amplifies a fragment beginning upstream of the recombination (and is therefore absent from the plasmid itself) and ends in the plasmid sequence. This fragment is expected to be of 925bps in length. Upon genomic integration, the entire plasmid will integrate into the genomic locus. The 3' arm amplifies a fragment that starts at the very end of the plasmid (just upstream of the recombination arm) and ends just downstream of the gene (a position which is absent from the plasmid). Since the exact position of double strand break can only be estimated, it is not possible to give an exact prediction of size. Both 5' as well as 3' PCR products require therefore integration of the plasmid and should not be amplified from the parental 3D7 gDNA. The "locus" starts 5' and ends 3' of the recombination arm and should therefore only be detectable in the parental parasite.

Figure 16: Validation of genomic integration by PCR

(a) PCRs spanning the 5', 3' and genomic loci of targeted var Pf3D7_0421300 in the transfected cell lines and the parental 3D7. Presence of bands in the 5' and 3' reactions validate correct integration of the plasmid into the targeted locus. Loss of the genomic locus proves complete integration in the entire population without wild type escape. PCR was positive for 5' and 3' product, but retained the genomic locus. (b) Table indicating the predicted sizes of the PCR products. Length of 3' arm can only be given as approximation as exact position of integration is unknown.

Retention of the endogenous locus in the transfected line has been observed and discussed in chapter 3.2.1. In summary, it might be due to the high sequence similarity of *var* gene loci, especially 3' of the stop codon (primer G_rev) which causes amplification of a different locus. To further investigate whether the construct integrated into the correct locus, the 5' PCR product was purified and sequenced, confirming integration into the correct site.

3.2.10. PfEMP1-GFP fusion protein is expressed and presented on the surface of the infected red blood cell

Correct expression and export of the full-length protein was validated by western blot analysis of saponin fractionated parasites (Figure 17a). Late stage parasites were enriched on a percoll gradient and treated with 0.05% saponin to separate the infected red blood cell into the parasite and host fractions as discussed in chapter 3.2.2. A band of the expected size of 279kDa (green arrow) is detectable in both fractions, confirming expression of the fusion protein as well as export into the host cell. Even though a band of the expected molecular weight is detected both the parasite internal as well as the exported (Host) fraction, an additional band of slightly more than 100 kDa in size is also detected in the host. This size would correspond to the PfEMP1ATS domain and GFP and could result from proteolytic cleavage during the saponin fractionation. Degradation of the entire extracellular part of PfEMP1 without trypsin treatment could be seen for one, but not all, of the “mini-PfEMP1” described before (Melcher et al., 2010).

Figure 17: Western blot analysis of PfEMP1 knock-in cell line

(a) Late stage parasites were enriched on a percoll gradient and extracted from the host red blood cell by saponin lysis, resulting in "Parasite" fractions of the purified parasite and "Host" fractions including the red blood cell membrane, membranous compartments within the host and its cytosol. Plasmodium aldolase, a parasite internal protein, served as loading control. The tagged PfEMP1 is present in both fractions (green arrow), suggesting correct expression of the fusion protein and export into the host cell. Band of 100kDa in the host fraction possibly corresponds to the PfEMP1 ATS domain coupled to the GFP tag. (b) Surface trypsinization of 0421300.GFP parasites. Late stage parasites were enriched on a percoll gradient and subjected to mild tryptic digest. Integral membrane proteins were purified by sequential detergent extraction. Trypsin treatment caused complete cleavage of the red blood cell surface protein glycophorin C (red arrow). Appearance of a 100kDa band after trypsin treatment indicates partial cleavage of the fusion protein (green arrow). (c) Table indicating predicted protein weight in kDa.

To validate that the protein is properly exported onto the surface of the infected red blood cell, live parasites were subjected to tryptic surface digest (Figure 17b). Parasites were first enriched on a percoll gradient and subsequently exposed to trypsin. After detergent extraction of integral membrane proteins, samples were analysed by western blot. Glycophorin C, an integral membrane protein of the red blood cell, served as positive control for successful digest (Furthmayr, 1978). Since the antibody against Glycophorin C detects the internal part of the protein, persistence of a smaller band shows that the cells remained intact during the treatment. After the digest, Glycophorin C underwent a 15kDa size shift, corresponding to the surface exposed part of the protein (red arrow). Pf3D7_0421300.GFP shows the characteristic multi-band pattern around 250kDa that has been shown for PfEMP1 before (Oberli et al., 2016; Waterkeyn et al., 2000). After treatment with trypsin, Pf3D7_0421300.GFP showed an additional band of slightly more than 100 kDa (green arrow), corresponding to the size of the PfEMP1 ATS domain tagged to GFP (83kDa ATS + 31kDa GFP). Partial cleavage of PfEMP1 has been described before and results from incomplete export of the protein onto the surface of the infected red blood cell (Waterkeyn et al., 2000). Another band of unknown origin can be detected at approximately 90kDa. This data strongly suggests that some of the tagged protein is exposed on the surface of the infected red blood cell.

The band of the trypsin cleaved PfEMP1 appears to be of the same size as the additional band observed in Figure 17a. The samples analysed were generated in different ways, making direct comparison difficult. It can therefore only be speculated what the origin of the additional band is. Smaller cleavage products of GFP tagged PfEMP1 have been shown before, possibly suggesting degradation of the fusion protein by the parasite (Melcher et al., 2010). This would suggest cleavage of the protein due to unknown mechanisms. Considering that the smaller sized band could only be detected in the parasite internal fraction, it could be speculated that the GFP causes export hinderance, resulting in breakdown of the protein.

3.2.11. Immunofluorescence analysis of PfEMP1.GFP confirms export and association with Maurer's Clefts

Subcellular localization of the protein was assessed by immunofluorescence analysis using the same markers as previously discussed (Figure 18). In short, cells were fluorescently stained for GFP and either EXP2 (which stains the parasitophorous vacuolar membrane), sEMP1 (a Maurer's Cleft resident protein) or Gap45 (part of the glideosome) (Baum et al., 2006; Dietz et al., 2014; Johnson et al., 1994). As suggested in the previous section, the tagged PfEMP1 is exported beyond the parasite and remains as punctates all over the infected red blood cells. This pattern is highly similar to the previously described GFP tagged PfEMP1 proteins (Melcher et al., 2010). In early stages, the protein overlaps with sEMP1, confirming the predicted export through the Maurer's clefts. Within merozoites, the protein is visible all throughout the cell. It is unclear what the role of the protein within the merozoite is. Antibodies targeting STEVOR in merozoites can inhibit invasion (Niang et al., 2014; Wichers et al., 2019). The investigated STEVOR could be either rhoptry localized or more dispersed, as is observed for 0421300.GFP. It could be speculated that the tagged PfEMP1 serves a similar role, even though experimental evidence for this assumption is lacking at this point.

Figure 18: Localization of PfEMP1 0421300.GFP in asexual stages by immunofluorescence assay

Parasites were fixed using methanol (trophozoite) or PFA (merozoite). Protein localization was determined by co-localization with EXP2 (PVM), sEMP1 (Maurer's Cleft) and Gap45 (glideosome). DNA was stained with Hoechst33342. The fusion protein Pf3D7_0421300.GFP remains to large parts within the parasite. The exported parts of the proteins localizes to dots within the host cell and at its periphery (green arrow). The exported pr otein partially co-localizes with Maurer's Clefts. It is present all throughout the merozoite.

In summary, the SLI knock-in method has been extended to the *var* multigene family and utilized to force expression of the *var* Pf3D7_0421300, a previously unstudied gene. Expression of the protein was strongest at 18 hours post invasion and drastically dropped at the later time points, coinciding with the published peak of expression. The GFP tagged protein was expressed at full length and exported beyond the boundaries of the parasite, where is partially overlapped with the Maurer's Clefts marker sEMP1. Furthermore, presentation of the protein on the surface of the red blood cell could be confirmed by mild tryptic digest of live trophozoites. The localization of the protein within the cell showed a striking resemblance of a previously described GFP tagged PfEMP1 (Melcher et al., 2010). These findings suggest that expression of the protein is not altered by our knock-in method but that native conditions were preserved.

3.2.12. Expanding the knock-in method to the *rif* family

The final multigene family targeted are the *rif*. Structurally similar to *stevor*, they have recently been shown to mediate the host innate immune response (Saito et al., 2017). There is ongoing debate surrounding the mutual exclusive control within this multigene family. Different experimental approaches were applied to answer this question, leading to inconsistent results. Phenotypic enrichment could lead to the monoallelic expression of one gene, but experiments utilizing *rif* introns or 5' UTR could not show any degree of mutual exclusive control (Araujo et al., 2018; Goel et al., 2015; Witmer et al., 2012). The advantage of the SLI method is that it results in the targeted gene remaining under the control of its endogenous promoter, making this approach suitable to solve this question. Two genes were chosen for expression: Pf3D7_1254800 and Pf3D7_1101100. Both genes are considered A-type RIFIN.

In contrast to the *var* gene, which was chosen at random among those that have not been studied before, the *rif* of choice have been associated with immune suppression. Pf3D7_1254800 has been shown to directly interact with LILRB1 and inhibit NK-cells expressing the receptor. Pf3D7_1101100 binds to LAIR1, another receptor on the surface of peripheral mononuclear cells.

3.2.13. Validation of SLI integration into the targeted locus

The constructs to knock in Pf3D7_1254800 or Pf3D7_1101100 were created analogous to their *var* counterpart. In summary, a 750 base-pair recombination arm was cloned between the NotI and MluI sites of pSLI, resulting in the excision of the 2xFKBP domains present in the original plasmid. This would result in tagging of the *rif* with GFP.

Figure 19: Validation of genomic integration by PCR

PCRs spanning the 5', 3' and genomic loci of targeted rif Pf3D7_1101100 (a) and Pf3D7_1254800 (b) in the transfected cell lines and the parental 3D7. Presence of bands in the 5' and 3' reactions validate correct integration of the plasmid into the targeted locus. Loss of the genomic locus proves complete integration in the entire population without wild type escape. Both cell lines are positive for 5' and 3' product, but only 1101100.GFP lost the endogenous locus. (c) Table indicating the predicted sizes of the PCR products. Length of 3' arm can only be given as approximation as exact position of integration is unknown.

Integration was confirmed by PCR using gDNA of the transfected cell line and parental 3D7 (Figure 19). Primer pairs were chosen as discussed in chapter 3.2.1. In short, the 5' arm amplifies a fragment beginning upstream of the recombination (and is therefore absent from the plasmid itself) and ends on the plasmid. Upon genomic integration, the entire plasmid will integrate into the genomic locus. The 3' arm amplifies a fragment that starts at the very end of the plasmid (just upstream of the recombination arm) and ends just downstream of the gene (a position which is absent from the plasmid). Both 5' as well as 3' PCR products require therefore integration of the plasmid and should not be amplified from the parental 3D7 gDNA. The "locus" starts 5' and ends 3' of the recombination arm and should therefore only be detectable in the parental parasite.

Integration PCR into the Pf3D7_1101100 locus showed the expected results with amplification of a 906bps fragment of the 5' arm and an approximately 800 bp fragment of the 3' PCR product. Both products are only present in the transfected cell line, which simultaneously lost signal of the endogenous locus of 931bps. Integration into the Pf3D7_1254800 was confirmed by the presence of bands of 876 and approximately 880bps for the 5' and 3' PCR products, respectively. The transfected cell retained 893bps signal of the endogenous locus, as was previously seen in case of Pf3D7_0102100, Pf3D7_1149900 and Pf3D7_421300 tagging. As in the previous cases, the PCR might amplify the locus of a different but highly similar locus.

3.2.14.RIFIN.GFP fusion proteins are expressed but only one is exported

Correct expression and export of the full-length protein was validated by western blot analysis of saponin fractionated parasites (Figure 20). Late stage parasites were enriched on a percoll gradient and treated with 0.05% saponin to separate the infected red blood cell into the parasite and host fractions as discussed in chapter 3.2.2. A band of the expected size of 69.8kDa (1101100.GFP) or 66.6kDa (1254800.GFP) (green arrow) is detectable in both parasite lines, confirming expression of the fusion protein. Only 1101100.GFP however is exported into the host fraction while 1254800.GFP remains entirely within the parasite. According to sequence predictions, both proteins are considered A-type RIFIN and should therefore expected to be exported into the host cell (Bachmann et al., 2015; S. A. Kyes et al., 1999). Considering that only of the two proteins remains parasite internal, it is unlikely that the GFP tag inhibits protein export. However, Pf3D7_1101100 showed an additional, parasite internal band of about 30kDa molecular weight, potentially corresponding to free GFP. It is an observation similar to the additional band described for the PfEMP1 0421300.GFP in chapter 3.2.10. Taken that into account it is possible that the large tag interferes with export, even though it does not entirely inhibit it. No smaller fragments could be detected with the much smaller 3.HA tag that was utilized in the *stevor* knock in (Chapter 3.2.2.). One way to show that the GFP indeed causes degradation of the protein within the parasite would be tag the same *rif* with a 3HA tag.

Figure 20: Western blot analysis of RIFIN knock-in cell lines

(a) Late stage parasites were enriched on a percoll gradient and extracted from the host red blood cell by saponin lysis, resulting in “Parasite” fractions of the purified parasite and “Host” fractions including the red blood cell membrane, membranous compartments within the host and its cytosol. Plasmodium aldolase, a parasite internal protein, served as loading control. The tagged RIFIN are both expressed at their predicted size (green arrows). Only 1101100.GFP is present in both fractions, suggesting export into the host cell. Band of 30kDa in the host fraction of 1101100.GFP possibly corresponds to free GFP. (b) Table indicating predicted protein weight in kDa

3.2.15. Immunofluorescence analysis of RIFIN.GFP confirms that only Pf3D7_1101100 is exported

Subcellular localization of the proteins was assessed by immunofluorescence analysis using the same markers as previously discussed (Figure 21). In short, cells were fluorescently stained for GFP and either EXP2 (which stains the parasitophorous vacuolar membrane), sEMP1 (a Maurer’s Cleft resident protein) or GapM / Gap45 (1101100.GFP / 1254800.GFP respectively) (both are part of the glideosome) (Baum et al., 2006; Dietz et al., 2014; Johnson et al., 1994).

Pf3D7_1101100 showed a localization similar to the PfEMP1 protein described earlier (Chapter 3.2.11.). GFP signal could be detected both in the parasite as well as in the host, where it localized to very few punctates. Even though it did not form the ring structure at the red blood cell periphery that has been described for certain members of A-type RIFIN class before, RIFIN with a similar localization haven been shown to be surface exposed (Goel et al., 2015). The protein only appeared from mid trophozoite

stage onwards. Considering that at this stage sEMP1 stains almost the entire cell, co-localization between the two proteins cannot be evaluated. Within merozoites, the protein is visible all throughout the cell, again resembling the pattern observed for the PfEMP1 0421300.GFP. This staining pattern as well has previously been associated with RIFINs (Bachmann et al., 2015).

Contrasting, 1254800.GFP remains entirely within the parasite, confirming the western blot results. It is present within each individual merozoite, where it is present throughout most of the cell and an additional distinct spot. Considering that it is not exported, no co-staining with sEMP1 was performed. Considering that the protein is grouped into A-type RIFIN, it is surprising that the protein remains parasite internal. RIFIN grouping though is based on the presence of a 25 amino acid insert, which has been confirmed with a small subset of genes. There has never been a large screen of *rif* performed to rule out exceptions from the predicted localization. Since the GFP apparently does not entirely inhibit export (RIFIN 1101100.GFP) or surface presentation (PfEMP1 0421300.GFP) of other surface antigens, it is likely that the parasite internal localization is not just an artefact due to the large tag.

Figure 21: Localization of 1101100.GFP and 1254800.GFP in asexual stages by immunofluorescence assay

Parasites were fixed using methanol (trophozoite) or PFA (merozoite). Protein localization was determined by co-localization with EXP2 (PVM), sEMP1 (Maurer's Cleft) and GapM (a) or Gap45 (b) (glideosome). DNA was stained with Hoechst33342. (a) The fusion protein Pf3D7_1101100.GFP remains partially within the parasite, but shows export to spots within the host red blood cell. The exported parts of the protein partially co-localize with Maurer's Clefts. It is present all throughout the merozoite. (b) 1254800.GFP remains entirely within the parasite and shows no export into the host cell. It is present in most of the merozoite but shows an additional distinct spot.

3.3. Epigenetic regulation within STEVOR multigene family

Despite the large body of information available on the epigenetic control mechanisms underlying *var* gene expression, little is known for the *stevor* multigene family. While most available information is based on overexpression of *stevor* 5' untranslated regions, there is to our knowledge no published study on the influence of histone tail modifications on *stevor* expression. In order to shed light on this topic, Tatenda Mushunje investigated the presence of certain histone modifications surrounding the ATG site of different *stevor* in their active and silent state (Tatenda Mushunje PhD thesis, manuscript in preparation). By comparing different clones with unique expression patterns, he could prove that active *stevor* are characterized by H4 and H3K9 acetylation. The genomic locus of inactive *stevor* is more variable, showing occupation by H3K9 trimethylation to varying degrees and H3K36 trimethylation only for a subset of genes. Even though this work provides first insights into the impact of histone modifications on *stevor* expression, the results are based on comparison of the genomic loci of two entirely independent clones. It is unknown whether these changes are specifically due to activity of the locus or whether the clones show larger epigenetic differences. The knock-in method established in this work provided the means to study the genomic locus of the same parasite clone in an "on" and "off" state. The knock-in parasites presented in this work maintain their expression due to the G418 drug pressure which forces expression of the targeted gene. Cultured parasites switch their *stevor* expression pattern over time. Removal of the drug should therefore result in a parasite culture which stopped expression of the targeted *stevor*. Re-supplementation of G418 would force the parasite to resume expression of the targeted gene, thus providing a method to monitor the histone modification pattern at a specific genomic locus in both states.

3.3.1. Knock-in of *stevor* causes mutual exclusive expression

Unlike *var*, little is known about mutual exclusive control among the *stevor* multigene family. Expression of 1-3 genes per parasite has been reported before (Lavazec et al., 2007; Niang et al., 2009). Furthermore, parasites can stop *stevor* expression entirely in culture (Blythe et al., 2008). It was unclear whether knock-in of one particular gene would cause mono-allelic expression or upregulation of

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several *stevor* at once. In order to evaluate the impact of *stevor* knock-in on overall *stevor* expression, qRT-PCR of all 40 annotated genes in the 3D7 reference genome was performed (Figure 22).

Only the targeted *stevor* Pf3D7_1149900 showed high levels of expression (Black arrow). Only two other genes (Pf3D7_0617600 and Pf3D7_0901600) showed detectable albeit very low levels of expression. This suggests that *stevor* expression is either mutually exclusive or that the parasite population went through a selective bottleneck (possibly during the G418 selection step) which could have eradicated almost the entire population. The emerging population would therefore have characteristics of a clonal selection such as distinct multigene expression patterns. This theory would also explain the almost monoallelic *var* expression observed. Furthermore, almost exclusive expression of the targeted gene suggests that the SLI constructs specifically integrated into the correct locus. Unspecific integration would likely lead to expression of all modified loci simultaneously due to the selective pressure on the neomycin resistance.

Figure 22: Quantitative real time PCR of all 40 *stevor* in the Pf3D7_1149900 knock-in cell line
RNA of 1149900.3HA was harvested 18 hours post invasion and cDNA synthesized. Quantitative RT-PCR on all 40 annotated *stevor* in the 3D7 reference genome was performed and normalized to serine tRNA ligase. Relative mRNA was calculated. A full list of primers used in this experiment can be found in chapter 2.5, table 3. The targeted *stevor* was confirmed to be the main transcript. Expression for all other *stevor* was either not detectable or at very low levels.

3.3.2. *Stevor* expression is controlled by a class III histone deacetylase

In order to generate a knock-in cell line that stopped expression of the targeted gene, 1149900.3HA was taken off G418 pressure for several months. Without presence of G418, parasites do not require expression of the neomycin resistance marker, allowing them to reduce and finally stop expression of the knock-in *stevor*. Expression of the fusion protein was controlled by immunofluorescence analysis. It was expected to observe a heterogenous culture of parasites without drug pressure. Those with reduced expression of the tagged STEVOR would have lower signals for the HA tag, while those that entirely stopped expression would show no HA signal at all. Since switches in expression appear to happen at a certain percentage at every cycle, only a small subpopulation of cells would initially cease to express the targeted gene. It was therefore necessary to control expression at a single cell level using immunofluorescence analysis instead of population basis such as western blot.

Even after prolonged periods, all parasites were positive for HA signal (data not shown). An alternative approach to change the epigenetic landscape is treatment of the parasites with drugs that inhibit deposition, removal or readout of histone tail modifications. Treatment of parasites with histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA) has been shown to cause drastic changes in expression patterns (Andrews et al., 2012). This “epigenetic reset” could cause a shift in *stevor* expression.

Parasites were treated with 50nM TSA for 3 cycles from 6 to 30 hours post invasion. Each treatment reduced parasitemia by about 90%. Parasites were harvested at 24 hours post invasion the cycle after finishing the treatment and RNA was extracted. Expression of three different *stevors* was analyzed by qRT-PCR (Figure 23). Compared to the untreated cells (1149900_initial), expression of one *stevor* remained low (Pf3D7_0631900), one increased (Pf3D7_1000800) and the targeted gene (Pf3D7_1149900) showed slightly reduced expression. In light of the drastic changes in expression observed for core genes in the previously mentioned study, these results suggest that treatment with the HDAC inhibitor TSA does not change the *stevor* expression pattern significantly. One thing of note is that the histone deacetylase PfSIR2, which is essential for correct *var* and *rifin* expression, is a class

III deacetylase (Gregoret, Lee, & Goodson, 2004). TSA is an inhibitor specifically for class I and II but not class III (Vanhaecke, Papeleu, Elaut, & Rogiers, 2004). Since TSA treatment only shows minimal effect in *stevor* expression, it is likely that either PfSIR2 or another, so far unidentified class III histone deacetylase is important for correct *stevor* expression.

Figure 23: Treatment of *stevor* knock-in cell line with TSA shows only minimal effect on *stevor* expression

Pf3D7_1149900 knock-in parasites were treated with 50nM TSA for three cycles from 6 to 30 hours post invasion. RNA was extracted 24 hours post invasion after the last treatment cycle. Expression of three different *stevor* was analysed. One *stevor* remained low, one *stevor* showed slight upregulation and the targeted *stevor* downregulation. These changes in expression were lower than the previously described effect of TSA treatment on parasites.

3.3.3. Single cell cloning of 1149900.3HA yields parasites that switched *stevor* expression

An alternative approach to generate parasites in a *stevor* “off” state was single cell cloning by limited dilution. The parental cell line was cultured without G418 pressure for extended periods of time and subjected to single cell cloning. As mentioned in 3.3.2., resistance to G418 is only maintained while the gene of interest is expressed. Removal of G418 allows the parasite to return to normal *stevor* switch rates, similar to what is observed with *var*. Considering the lengthy period of time required for single cell cloning and the stress associated with it, cloning would identify randomly switched parasites. A total of 9 clones were obtained and fusion protein expression was validated by western blot (Figure

24a). Out of the 9 clones, 4 (C5, C6, F7 and G2) apparently stopped expression of the targeted protein while 4 (B6, B7, D7 and F2) showed expression to varying degrees. Due to very weak signals of the loading control, no conclusions can be drawn for the remaining one (F7). The clones C5 and G2 were chosen for further analysis. Expression of the protein could be recovered in both clones by reapplication of G418, showing that disappearance of the fusion protein was indeed due to a switch in expression and not loss of genomic integration (Figure 24b).

Figure 24: Single cell cloning yields parasites in reversible *stevor* “off” state
(a) Pf3D7_1149900 knock-in parasites were taken off G418 drug pressure and subjected to single cell cloning. Resulting clones were screened for Pf3D7_1149900.3HA expression by western blot. The clones C5 and G2 (black arrows) were chosen for further characterization. (b) Expression of the targeted *stevor* can be reactivated by supplementation of G418.

Finally, both clones were tested for their expression of all 40 *stevor* as well as 5 *var* and 5 *rif* (Figure 25). Both clones stopped expression of Pf3D7_1149900, confirming the western blot results. Unlike the parental pool, both of them stopped expression of the *rif* Pf3D7_1149800, a gene which is directly adjacent to the targeted *stevor*. Both clones show expression of a different, untargeted *stevor* (clone G2 expresses Pf3D7_1040200 while clone C5 expresses Pf3D7_0832000). Both clones express the same *var* (Pf3D7_0617400) as the parental line, albeit at drastically elevated levels.

Figure 25: Single cell cloning of 1149900.3HA yielded clones expressing different stevor

qRT-PCR of all 40 stevor genes, 5 var and 5 rifin in the 1149900.3HA cell line and the two “off” clones. Both “off” clones switched expression from the initially targeted Pf3D7_1149900 (black arrow) to a different stevor and stopped expression of the co-regulated rifin Pf3D7_1149800. Expression of the dominant var Pf3D7_0617400 increased compared to the parental strain.

3.4. Regulatory mechanisms within and between multigene families

The mutual exclusive control among *var* is well established and has been proven in numerous studies in field isolates as well as laboratory strains (Scherf et al., 1998). Expression of one gene in the family results in silencing of the others, ensuing expression of only one gene from that family at a time. Little is known about the control mechanism among *stevor* and *rif*. Studies have shown expression of one or few *stevor* per parasite, while a larger amount of different *rif* seem to be expressed (Blythe et al., 2008; Lavazec et al., 2007; Petter et al., 2008). On the other hand, phenotypic enrichment appears to cause monoallelic expression of *rif* (Goel et al., 2015). Since the knock-in method forces expression of a given gene of one multigene family, complete silencing of other members of that multigene family should be observed if the gene family is under mutual exclusive control.

Furthermore, mechanistic insights into the cross-talk between multigene families are still lacking. While presence of a “titratable factor” which controls overall variant antigen expression has been described earlier, another study questioned its’ impact on *stevor* and B-type *rif* expression (Dzikowski

et al., 2006; Howitt et al., 2009). One of the advantages of the described SLI knock-in of multigene family members over episomal overexpression is the inclusion of epigenetic control mechanisms. The genes are expressed under their endogenous promoter instead of constant overexpression. Mutual exclusive control would be maintained after forcing expression of a given gene. Furthermore, cross-talk between families would remain active. If cross-talk within and between the families exists, expression of other variant genes should adjust. Forcing the expression under its endogenous promoter ensues involvement of upstream untranslated regions as well as intronic sequences, both of which are factors involved in *var* regulation.

In Chapter 3.3.1. it was established that knock-in of a *stevor* causes monoallelic expression of the targeted gene, similar to what is expected from *var*. However, while there are only 40 annotated *stevor* genes, *var* (60) and *rif* (150) are too numerous for qRT-PCR validation of every single member. Microarray analysis was therefore applied to assess the impact of *stevor*, *var* and *rif* knock-in on global multigene expression patterns. This would potentially answer three questions: is SLI based knock-in maintaining the epigenetic control mechanisms, are *stevor* and *rif* under any kind of mutual exclusive control and whether is multigene family transcending control in *P. falciparum*.

Global changes in expression upon knock-in of a *stevor*, *var* or *rif* were assessed by microarray analysis (Figures 26, 27 and 28 respectively). Expression in the Pf3D7_1149900, Pf3D7_0421300 and Pf3D7_1254800 knock-in cell lines was compared to the parental 3D7 control. Parasite samples were harvested from a highly synchronous culture at 18, 24 and 30 hours post invasion. These timepoints were chosen to cover peak expression of *var*, *rif* and *stevor* multigene families. As reference pool, parasite samples were harvested from 3D7 at 6hour intervals, covering the entire life cycle of the parasite. RNA was extracted using TRIzol, followed by cDNA synthesis and SMART amplification as was described before (Bozdech et al., 2003). Sample cDNA and pool cDNA were stained with Cy5 and Cy3 respectively, followed by hybridization of sample cDNA against the pool cDNA. Data acquisition and analysis was performed using GenePix Pro 3 and the $\log_2(\text{Cy5/Cy3})$ ratio calculated. Relative changes in the expression of the three multigene families are given as $\log_2$ fold changes of the sample compared

to the reference pool. The first three columns of each heatmap represent changes in expression in the parental 3D7 control while the second three columns represent the knock-in cell line. Each row shows expression changes of *stevor* (Figure 26, 27, 28a), *var* (Figure 26, 27, 28b) and *rif* (Figure 26, 27, 28c). Datapoints with no value due to their intensity being below threshold are shown as black since they are considered as not expressed for the purpose of this analysis.

Finally, a small subset of genes (5 *var*, *stevor* and *rif*) were chosen for qRT-PCR validation (Figure 26, 27, 28d, multigene family is indicated by v, r or s respectively). Knock-in parasites were grown in the blood of three different donors in parallel for four cycles. All three cultures were handled in parallel and harvested at the same time to ensure stage specificity. Parasites were collected at 18 hours post invasion. Expression was normalized to serine tRNA ligase (Pf3D7_0717700) and relative expression calculated.

3.4.1. Knock-in of a *stevor* changes the overall multigene expression pattern

The degree of control within the *stevor* multigene family is still not fully understood. It is commonly accepted that each parasite expresses 1–3 genes at any point in time (Lavazec et al., 2007; Niang et al., 2009). Expression can be lost in culture and in splenectomised patients, suggesting that host immune pressure is essential to maintain expression (Bachmann et al., 2009a). One experimental attempt to study the degree of mutual exclusive control was expression of a paired intron and measure the changes in *stevor* expression (Witmer et al., 2012). The same approach, when applied to *var*, caused downregulation of endogenous *var* loci, highlighting the importance of intron derived non-coding RNAs in *var* transcription. No significant effect on *stevor* expression could be detected, suggesting, if present, a different method of control. Episomal expression of *var* promoters caused downregulation of *stevor* expression, showing that *stevor* are part of a multigene family transcending control mechanism (Dzikowski et al., 2006). One shortcoming of all of these studies is the artificial approach implemented to study locus activity. By forcing the parasite to express a specific gene under its endogenous promoter, the underlying control mechanism will likely stay in place, creating a platform to study the *stevor* expression dynamics.

Figure 26: Analysis of multigene expression pattern upon *stevor* knock-in

Global changes in expression of *stevor*, *var* and *rif* upon knock-in of Pf3D7_1149900 were analysed using microarray. Shown are changes in *stevor* (a), *var* (b) and *rif* (c) expression. RNA was harvested for 3D7 and 1149900.3HA at 18, 24 and 30 hours post invasion. Green indicates downregulation, red upregulation as compared to the reference pool RNA. Data is given as log fold change. Datapoints with no value (intensity below threshold) are shown as black since they are considered as not expressed for the purpose of this analysis. (a) The 3D7 control expresses several different *stevor*. Upon knock-in, the targeted *stevor* becomes strongly upregulated (black arrow). Several other genes are upregulated to lower degree. (b) While the 3D7 control shows expression of several *var* at the 18 and 24 hours timepoints, the knock-in cell line shows strong upregulation for only one *var* (Pf3D7_0617400, black arrow) (c) Both cell lines express a wide panel of *rif*. Considering that several genes retained their expression (green arrow), change in overall expression was less dramatic as compared to *stevor* and *var*. The genomic locus of the *rif* with the highest degree of upregulation (Pf3D7_1149800, indicated with black arrow) is located directly adjacent to the targeted *stevor*. (d) qRT-PCR validation of microarray results. RNA was harvested 6 weeks after the microarray analysis and grown in the blood of three different donors. V, r and s behind the geneID indicate *var*, *rif* or *stevor* multigene family, respectively. Results confirm microarray with the exception of Pf3D7_0700400_s which showed upregulation in the microarray but was not detectable in the qRT-PCR validation.

Targeting the *stevor* Pf3D7_1149900 caused strong upregulation of the gene compared to the reference pool (Figure 26a, black arrow). Furthermore, it caused an overall change in the *stevor* expression pattern. While the 3D7 control expresses several *stevor* at intermediate levels, the knock-in cell line shows high upregulation of the targeted gene and low levels of expression of several other *stevor*.

A similar shift in overall expression pattern was observed among the *var* (Figure 26b). The unselected 3D7 cell line expresses several different genes at intermediate levels with *var2csa* (Pf3D7_1200600) being the strongest. The *stevor* knock-in cell line on the other hand expresses only one *var* (Pf3D7_0617400, black arrow) at high levels. Considering that it is located on a different chromosome than the targeted *stevor*, it is unknown whether this seemingly monoallelic expression is due to a

population bottleneck during the selection process or a potential cross-talk between the two multigene families. It is of note that the same gene showed highly elevated levels of expression after subcloning of the knock-in cell line. The gene is, unlike most other multigene family loci, located chromosome internal. These loci have previously been associated with lower switch rates, possibly explaining the consistent expression and arguing for a bottleneck effect on the population (Frank et al., 2007).

Among the *rif*, the effect of *stevor* knock-in was less obvious than among the *var* (Figure 26c). Both the parental as well as the knock-in cell line express a wide array of different *rif*. Furthermore, many genes show similar expression patterns in both cell lines (e.g. Pf3D7_1000600, Pf3D7_0700500, Pf3D7_1400300, Pf3D7_0401600, green arrows). The one *rif* with the strongest degree of upregulation is Pf3D7_1149800 (black arrow). The gene is located 1.9kbps upstream of the targeted *stevor* Pf3D7_1149800 in a head to tail manner. Considering the very high degree of upregulation and the proximity of the two loci, it is tempting to speculate about cross talk between the two multigene families. On top of that, similar effects will be described (Chapter 3.4.2.) and discussed (Chapter 3.4.4.) further on.

Among the five tested *var*, Pf3D7_0617400 showed the highest mRNA abundance, confirming the microarray data and mimicking the results from chapter 3.3.1. The other four genes showed either very low or are below detectable levels of expression. Similarly, only one of the tested *rif* (Pf3D7_1149800) displayed high mRNA abundance according to the qRT-PCR. This is in line with microarray analysis which only showed upregulation for this *rif* among the five chosen for qRT-PCR validation. Finally, the targeted *stevor* showed the highest degree of expression of the five tested. Surprisingly, Pf3D7_0700400, which was among the low expressing *stevor* according to the microarray, was not detectable at all. This observation is most likely due to the inherent difference between microarray and qRT-PCR and will be further discussed in a later on (Chapter 3.4.5.)

3.4.2. Targeting of Pf3D7_0421300 causes upregulation of *var* and *rif* in genomic proximity

Considering the tight mutual exclusive control within the *var* gene family, forcing the parasite to express a specific *var* of choice should alter the *var* multigene expression pattern within this transgenic parasite line. Without phenotypic enrichment, the parasite stochastically switches expression of its multigene repertoire after a given number of replications, thus giving rise to a culture which expresses many different members of the families. Phenotypic enrichment, for example by panning of parasites on endothelial cell lines, commonly enriches populations which express single or a small subset of *var* (Golnitz, Albrecht, & Wunderlich, 2008). If SLI based knock-in of a *var* maintains the epigenetic control mechanisms, only the targeted gene should be expressed while all other *var* are silenced.

Surprisingly, the knock-in cell line shows strong expression for only two *var*, Pf3D7_0421300 and Pf3D7_0421100 (Figure 27b, black arrow). The genes for the two upregulated *var* are oriented in a head to tail manner and separated by about 5.1kbp which include the non-coding RNA RUF6. This finding is similar to the upregulation of an adjacent *rif* described in the previous chapter. Furthermore, one *rif* (Pf3D7_0421500) in the knock-in cell line displays much higher levels of upregulation than the remaining ones (Figure 27c, black arrow). Analogous to Pf3D7_0421100, Pf3D7_0421500 is located in a head to tail manner to the targeted Pf3D7_0421300. Strikingly, *rif* Pf3D7_0421200, which is located 800bps downstream of the targeted *var* in a head to head fashion, was not upregulated (green arrow). Considering that histone modifications play a major role in *var* gene regulation, it is possible that active chromatin marks around the targeted gene make the entire genomic region accessible for RNA Pol II. This would therefore lead to upregulation of otherwise silenced members of multigene families.

The knock-in cell line and 3D7 both express a variety of different *stevor* as well as *rif* (Figure 27a and c). With the exception of the mentioned *rif* Pf3D7_0421500, the remainder of the two multigene families therefore show an expression pattern similar to the 3D7 control with a high diversity of expressed genes in a non-selected cell line.

Figure 27: Analysis of multigene expression pattern upon var knock-in

Global changes in expression of *stevor*, *var* and *rif* upon knock-in of Pf3D7_0421300 were analysed using microarray. Shown are changes in *stevor* (a), *var* (b) and *rif* (c) expression. RNA was harvested for 3D7 and 0421300.GFP at 18, 24 and 30 hours post invasion. Green indicates downregulation, red upregulation as compared to the reference pool RNA. Data is given as log fold change. Datapoints with no value (intensity below threshold) are shown as black since they are considered as not expressed for the purpose of this analysis. (a) The 3D7 control expresses several different *stevor*. Upon knock-in, the overall expression pattern switches to a different subset of genes. (b) While the 3D7 control shows expression of several *var* at the 18 and 24 hours timepoints, the knock-in cell line shows strong upregulation for only two *var*: the targeted Pf3D7_0421300 and Pf3D7_0421100 (black arrow). Both genes are oriented in a head to tail manner on chromosome 4, 5kbp apart. A few other genes appear to be slightly upregulated (e.g. Pf3D7_0533100, green arrow) (c) Both cell lines express a wide panel of *rif*. The *rif* with the highest degree of upregulation (Pf3D7_0421500, indicated with black arrow) is located directly adjacent to the targeted *var*. Another adjacent *rif* (Pf3D7_0421200, green arrow), shows no upregulation (d) qRT-PCR validation of microarray results. RNA was harvested 6 weeks after the microarray analysis and grown in the blood of three different donors. One of the replicates was excluded from final analysis due to strong differences in total mRNA abundance compared to the other two. V, r and s behind the geneID indicate *var*, *rif* or *stevor* multigene family, respectively. Results confirm microarray with the exception of Pf3D7_0533100_v, which showed upregulation in the microarray but was not detectable in the qRT-PCR validation.

A subset of the differentially controlled genes was chosen for further qRT-PCR validation (Figure 27d). While the results from two of the samples were highly similar, the results from the third sample differed strongly and was therefore excluded from the analysis. The difference arises from elevated expression of both *var* and variable expression of one *rif* (Relative expression of *var*_Pf3D7_0421100: 25.1, 6.8, 4.8; *var*_Pf3D7_0421300: 11.9, 4.5, 2.9; *rif*_Pf3D7_0421500: 0.6, 2.4, 1.0). Even though it can only be speculated as to why the expression differed so strongly, the questionable replicate was grown in blood close to its shelf life of 4 weeks, possibly changing expression dynamics. Considering the strict temporal control of *var* expression, few hours difference in effective age of the parasite can have a strong effect on mRNA abundance. At this point of the life cycle (ring to early trophozoite stage

transition), exact estimation of the parasite age using Giemsa stained thin blood smears is very inaccurate. Even though parallel culturing of the replicates was supposed to exclude age differences between them, a slightly shortened life cycle in the questionable replicate could potentially explain the differences in relative transcript abundance of the two *var*. Nonetheless, the results largely overlap with the results obtained from the microarray. They differ however in some details.

According to the microarray, the knock in cell line expresses several *var* genes besides the previously mentioned Pf3D7_0421100 and Pf3D7_0421300 at low levels, such as Pf3D7_0533100 (Figure 27b, green arrow). According to the validation, Pf3D7_0533100 is expressed below detection levels. This observation is most likely due to the inherent difference between microarray and qRT-PCR and will be further discussed in chapter 3.4.5.

3.4.3. *Rif* are not under mutual exclusive control

The impact of *rif* knock in on global multigene expression was assessed by microarray analysis of the Pf3D7_1254800 knock-in cell line (Figure 28).

Upon knock in, even though the expression pattern of individual multigene family members changed drastically, the overall pattern did not change compared to the 3D7 control. Within all three multigene families, the knock-in cell line expresses several members at the same time. Among the *stevor*, the two genes Pf3D7_1040200 and Pf3D7_1254100 (black arrows) show a similar expression pattern as the parental cell line. Other genes are either reduced (Pf3D7_0617600) or increased (Pf3D7_0114600) in their expression. The *var* display a similar behaviour. Several genes appear to be upregulated (such as Pf3D7_0532700 or Pf3D7_0533100, black arrows).

Finally, the targeted *rif* did not become the main transcript. Even though overall *rif* expression changed upon knock-in, the cell line still expresses a vast array of different genes. The targeted gene is overexpressed compared to the reference pool, but does not display the exceptionally high values observed upon *stevor* or *var* knock-in. Furthermore, Pf3D7_1254800 is already being expressed by the 3D7 control. Knock-in only increases expression at the 18 and 30 hours post invasion timepoints. These

observations are in line with previous findings which describe a lack of mutual exclusive control within the rifin multigene family (Petter et al., 2008).

Finally, the microarray results were validated by qRT-PCR validation (Figure 28d). The results largely overlap with the results obtained from the microarray. They differ however in some details. According to the microarray, the knock in cell line expresses several *var*, Pf3D7_0533100 being one of them. Pf3D7_0632500 (green arrow) on the other hand is considered not expressed according to the array. The qRT-PCR validation confirms expression of Pf3D7_0533100, but Pf3D7_0632500 display much higher levels of mRNA. Parasites were harvested for qRT-PCR validation several weeks after performing the microarray, potentially giving the parasite the opportunity to change its *var* expression. Expression of the targeted *rif* Pf3D7_1254800 as well as another upregulated gene (Pf3D7_0532900, green arrow) was confirmed in the qRT-PCR.

Figure 28: Analysis of multigene expression pattern upon *rif* knock-in

Global changes in expression of *stevor*, *var* and *rif* upon knock-in of Pf3D7_1254800 were analysed using microarray. Shown are changes in *stevor* (a), *var* (b) and *rif* (c) expression. RNA was harvested for 3D7 and 0421300.GFP at 18, 24 and 30 hours post invasion. Green indicates downregulation, red upregulation as compared to the reference pool RNA. Data is given as log fold change. Datapoints with no value (intensity below threshold) are shown as black since they are considered as not expressed for the purpose of this analysis. (a) The 3D7 control expresses several different *stevor*. Upon knock-in, the overall expression pattern switches to a different subset of genes. Highlighted is one gene with reduced (Pf3D7_0617600) and one with increased (Pf3D7_0114600) expression after knock-in. (b) The 3D7 control expresses several different *var*. Upon knock-in, the overall expression pattern switches to a different subset of genes. Black arrows indicate two upregulated genes (Pf3D7_0532700 and Pf3D7_0533100). Green arrow indicates an unaffected gene (Pf3D7_0632500). (c) Both cell lines express a wide panel of *rif*. The targeted *rif* Pf3D7_1254800 (black arrow) is among those with intermediate overexpression. Green arrow indicates another upregulated *rif* (Pf3D7_0532900). (d) qRT-PCR validation of microarray results. RNA was harvested 6 weeks after the microarray analysis and grown in the blood of three different donors. V, r and s behind the geneID indicate *var*, *rif* or *stevor* multigene family, respectively. Results confirm microarray with the exception of Pf3D7_0632500_v, which showed no expression in the microarray. The qRT-PCR validation indicates strong upregulation. This could potentially be due to a switch in *var* expression since the microarray analysis.

3.4.4. Variant surface antigens show family transcending co-regulation

It has been described before that the genomic loci of inactive multigene family members are occupied with heterochromatic marks, specifically histone tail methylation, making it inaccessible to the transcriptional machinery (Cabral, Fotoran, & Wunderlich, 2012; Chookajorn et al., 2007). In order to be accessible for RNA Pol II and consequently actively transcribed, among other factors, the chromatin has to be opened up.

Knock-in of the *var* Pf3D7_0421300 caused drastic changes in the expression of this multigene, as is expected considering the mutual exclusive expression within this family. While the parental 3D7 expresses several different *var*, the knock-in cell line only expresses two: the targeted one and

Pf3D7_0421100 which sits 5kbp downstream in a head to tail orientation (the genomic region surrounding Pf3D7_0421300 as well as the other four genes which will be discussed further on can be seen in Figure 29). We speculate that forcing the parasite to express a specific *var* opens up the chromatin at the genomic locus, causing upregulation of adjacent genes. This argument is further strengthened considering that the *rif* Pf3D7_0421500, which is upstream oriented in a head to tail manner to the targeted *var*, is upregulated as well. Expression of the *rif* Pf3D7_0421200, oriented in a head to head manner and located downstream of the targeted *var*, was not detectable, suggesting selectivity of this effect. A similar observation could be made when targeting of Pf3D7_1149900. Expression of the *rif* Pf3D7_1149800, also oriented in a head to tail manner and located 1.9kbp upstream of the targeted *stevor*, showed strong upregulation. Considering that Pf3D7_115000, a *rif* located 2kbp downstream in a head to tail manner to the targeted *stevor*, shows no change in expression complicates interpretation of our observations. No co-regulation could be observed in the Pf3D7_1254800 knock-in cell line, despite the presence of other *rif* in the vicinity of the targeted locus. After single cell cloning of Pf3D7_1149900 knock-in parasites, both clones show weak expression of the *stevor* Pf3D7_0617600 which is located 6.7kbp downstream of the *var* and oriented in a head to tail manner, resembling the same passive upregulation described.

A recent publication attempted to map the accessible genomic regions of two different clones of *P. falciparum* (Ruiz et al., 2018). The cell lines differed in their multigene expression patterns, providing suitable datasets to test the open chromatin hypothesis. The cell line 10G showed high levels of expression in 2 *var* and 3 *rif*. Out of those 5 genes, only one (PF3D7_0937800) was not in the genomic vicinity of one of the others. The high expressing of *var* Pf3D7_0426000 and the *rif* Pf3D7_0425900 are oriented head to head and 1.8kbp apart. The two *rif* Pf3D7_1100300 and Pf3D7_1100500 (head to head, 3kbp apart) are both highly upregulated, but not Pf3D7_1100400, which is located in between them. Taken together, there appears to be a pattern that high levels of expression can cause the passive upregulation of another gene in its close vicinity. This effect appears to be multigene family

transcending and is independent of orientation. Nothing is known so far about the mechanism underlying this cross talk.

Figure 29: Genomic area of 5 selected loci which show passive upregulation of adjacent multigene family members

Genomic loci surrounding members of multigene families that show passive upregulation of adjacent genes were extracted from PlasmDB. The top three genes (Pf3D7_0421300, Pf3D7_1149900 and Pf3D7_1254800) were knocked-in in this work and global expression was analysed by Microarray. The last two (Pf3D7_0426000 and Pf3D7_1100500) were part of a study which identified accessible genomic regions in different *P. falciparum* clones.

The hypothesis of chromatin accessibility is appealing, considering the inconsistency observed it cannot be the sole factor. A potential explanation could be differences in the 3' UTRs of the different genes, only allowing co-regulation of certain subsets. Non-coding RNA, produced during transcription of adjacent members of multigene families, could cause specific upregulation of other genes. Enhancers play an essential role on transcription in higher eukaryotes (T. K. Kim, Hemberg, & Gray, 2015). Furthermore, the importance of GC rich ncRNA or long ncRNA derived from *var* introns on expression is well established, even though evidence so far only depicts repressive effects (Deutsch et al., 2001; Dzikowski et al., 2007; Guizetti, Barcons-Simon, & Scherf, 2016). Finally, intergenic enhancer elements can produce bi-directional ncRNA capable of transcriptional control both in a cis manner as well as of adjacent genes (Ubhe, Rawat, Verma, Anamika, & Karmodiya, 2017). These enhancer elements are further segregated into housekeeping and virulence gene regulating clusters, providing a potential mechanistic explanation for the co-regulation observation described.

3.4.5. Limitation of microarray analysis in assessing multigene families

The signal detected in the microarray is fluorescently labelled DNA captured by a probe on the array chip. The signal of the actual sample is then compared to the signal obtained from the reference pool. The final data presented is therefore a relative increase or decrease of signal intensity between the sample and the pool DNA. Considering that the reference pool is an unselected culture, it is being assumed that the multigene expression pattern is stochastically distributed within the parasite population, covering all members from all three families. This would mean that each parasite expresses its unique subset of genes. Each gene would therefore only be expressed by a small subpopulation of parasites within the population. If this assumption is correct, the signal of members of multigene families in the reference pool would average within the population and could, depending on the peak expression, reach very low abundance. If this pool is compared to a culture which is enriched for expression of a certain gene, it should readily be detected as upregulated. This is in stark contrast to qRT-PCR which directly measures abundance of a specific DNA within the sample.

If this underlying assumption is not correct, as is potentially seen in the microarray analysis of Pf3D7_1254800 (chapter 3.8.3.), an expressed gene would be considered silent. In this example, the *rif* is already expressed at a certain level in the pool. Targeting of the gene with SLI ensured its expression, but most likely did not cause upregulation. Therefore, the fluorescent signal of the test sample and the reference pool would appear very similar in intensity and would therefore not be considered upregulated.

False positive results on the other hand can occur if a gene is expressed at very low levels in the pool and slightly elevated in the sample. After detection of the fluorescent signal and during analysis of the data, sample below a certain intensity is cut off and considered not detected. This detection limit however still includes low abundant DNA. If a gene is expressed at near undetectable levels in the pool, even very weak expression in the sample will be considered as strong upregulation. This can be seen in both 1149900.3HA and 0421300.GFP cell lines, which showed false positive expression of Pf3D7_0700400 and Pf3D7_0533100 respectively (chapter 3.4.1. and 3.4.2). Even though microarray analysis provides an overview of the multigene family expression pattern within a cell line, it is essential to verify the data by qRT-PCR to avoid false results.

3.5. Identification of intracellular interaction partners of surface expressed and internal STEVOR

While the intracellular interaction partners of PfEMP1 have been focus of extensive research, very little information is available on the anchoring of sVSA. It has been established that anchorage of PfEMP1 is essential to protect against the shear stress during sequestration (Y. Zhang et al., 2015). It is likely that small variant antigens would need a similar complex of proteins to maintain host cell ligand interactions under blood flow conditions. The STEVOR Pf3D7_0631900 appears to be interacting with the spectrin cytoskeleton of infected red blood cells in gametocytes and is phosphorylated by PKA (Naissant et al., 2016). STEVOR in general locate to the base of knobs in asexual parasites (Singh et al., 2017). These findings only address sexual stages or give indirect conclusion about their interacting

partners. It is not clear whether STEVOR are anchored directly to the red blood cell cytoskeleton or indirectly by interaction with another parasite protein which anchors the entire complex. Furthermore, it is unknown whether STEVOR specifically interact with proteins in the Maurer's Clefts. Considering that a number of STEVOR appear not to be displayed on the surface of red blood cells (Pf3D7_0102100 / Pf3D7_1149900 which are discussed in this work and PF3D7_1300900 / PF3D7_0300400 which have been described before), a potential role for these proteins within the host red blood cell appears likely (Bachmann et al., 2015). An in-depth analysis of interaction partners of different STEVOR could therefore identify new roles of small variant antigens in parasite biology. Two independent approaches were utilized to this end: co-immunoprecipitation and proximity biotin labelling.

3.5.1. Co-immunoprecipitation of STEVOR interaction partners

Co-immunoprecipitation utilizing 3HA tags have been utilized in numerous studies to identify protein interaction in *P. falciparum* (Baumgarten et al., 2019; Dietz et al., 2014; Mesen-Ramirez et al., 2016; Oberli et al., 2016; M. Zhang, Faou, Maier, & Rug, 2018). The two previously described proteins Pf3D7_0631900 (which is surface exposed) and Pf3D7_1149900 (which appears to remain within the Maurer's Clefts) were chosen for co-immunoprecipitation. Both proteins show a distinctly different final localization, and correct localization of Pf3D7_0631900 has been confirmed by others (Wichers et al., 2019). Furthermore, both are expressed at the expected size and inserted into the membrane utilizing the previously suggested central transmembrane domain (Bachmann et al., 2015). Due to their differential localization, the co-immunoprecipitation should yield new insights into the role of not surface exposed STEVOR and red blood cell anchorage of surface exposed STEVOR.

3.5.2. Optimizing conditions for anti-HA co-immunoprecipitation

Several conditions of the co-immunoprecipitation were optimized to identify the most advantageous setting (Figure 30). Considering that the focus of this experiment lies on proteins that are exported into the host, pull down efficacy was compared between saponin and RIPA lysed 1149900.3HA parasites (Figure 30a, b). While RIPA lyses the entire parasite, saponin at the conditions tested (0.05%

saponin in PBS for 5 minutes at room temperature) only solubilizes the red blood cell membrane and membranous compartments within the host cells, thus removing parasite internal proteins and reducing sample complexity. Late stage parasites were enriched on a percoll gradient and split into two equal fractions, one lysed with saponin and one with RIPA. The supernatants were subsequently incubated with anti-HA conjugated magnetic beads. Bound proteins were finally eluted with protein loading dye and analysed by western blotting. Input and elution were quantified and the percentage of total protein recovery after co-immunoprecipitation was calculated (Figure 30b).

Figure 30: Comparison of saponin and RIPA lysis of parasites for co-immunoprecipitation

(a) Western blot analysis of α -HA co-immunoprecipitation of RIPA or saponin lysed parasites. Late stage parasites were enriched on a percoll gradient and lysed using either RIPA or 0.05% saponin. The supernatant was incubated with α -HA conjugated magnetic beads. Input, Flowthrough and Elutions were probed for a variety of proteins. Only the tagged STEVOR could be detected in the final elution. (b) Percentage of captured protein in the two different lysis methods. Band intensity in input and elution was quantified and normalized to the amount of sample loaded onto the gel (10% of input and 100% of elution). Data given is the average of two western blots. (c) Table indicating the molecular weights of proteins that were probed for.

While both lysis methods solubilized a similar amount of protein, almost threefold the amount of protein was captured after RIPA lysis as compared to saponin. None of the other proteins that were probed for (including spectrin α which has been associated with STEVOR in gametocytes) could be detected in the elution of either sample.

Optimal bead to lysate ratio was validated by performing co-immunoprecipitation of three equal volumes of 1149900.3HA RIPA lysate with increasing amounts of beads. Binding capacity was compared by loading of the entire elution onto an SDS gel and subsequent western blot analysis (Figure 31). While both a 1:5 (bead slurry to lysate) and a 1:10 ratio appear to catch equal amounts of protein, 1:20 has a drastically reduced amount of protein in the eluted fraction. It is of note that a 1:20 ratio caused aggregation of the beads, which could possibly reduce binding capacity. Since the experiments were performed in parallel, the cause of aggregation is unclear. In order to avoid the risk of further aggregation, all subsequent experiments were therefore performed with a ratio of 1:10 bead slurry to parasite lysate.

Figure 31: Optimization of parasite lysate to bead slurry ratio.

Supernatant of RIPA lysed 1149900.3HA parasites was incubated with α -HA conjugated magnetic beads at the given ratios (bead slurry: parasite lysate). Total elution was loaded for western blot analysis. No differences between 1:5 or 1:10 could be detected. 1:20 appears to be insufficient for complete protein capture.

Finally, co-immunoprecipitation efficacy between the anti-HA (positive sample) and the anti-c-myc (negative control) conjugated magnetic beads was compared (Figure 32). 0631900.3HA parasites were, prior to RIPA lysis, treated with 20mM dithiobis(succinimidyl propionate) (DSP), a short range cross-linking agent. The lysate was split into two equal fractions and incubated with beads at a 1:10 ratio (Figure 32). The STEVOR protein could only be detected in the positive elution and not in the negative

control, confirming that the tagged protein binds specifically to the anti-HA antibodies and not the magnetic beads. In contrast to the previous co-immunoprecipitation, which was performed without cross-linking of proteins, spectrin α could clearly be detected in both elutions. Considering that the protein was present in both elutions, it is not an interaction specific to Pf3D7_0631900, but is likely artefact introduced by the cross-linking process. Furthermore, the previously STEVOR associated actin could not be detected in the elution of α -HA conjugated beads. Considering the weak staining seen on the western blot, it does not necessarily suggest that the STEVOR Pf3D7_0631900 does not interact with actin in asexual stage parasites. The lack of actin in the eluted fraction might be due to protein levels below detection limit.

Figure 32: Comparison of HA and c-myc antibody conjugated beads.

Supernatant of DSP treated, RIPA lysed 0631900.3HA parasites was incubated with either anti-HA or anti-c-myc

conjugated beads at a 1:10 ratio. Input, Flowthrough, washes 1-4 and elutions were probed for a variety of proteins. Tagged protein is only detectable after immunoprecipitation with anti-HA conjugated beads, confirming specificity of the co-immunoprecipitation. Spectrin α could be detected in the elution of both beads, suggesting that it is a contaminant protein rather than a specific interaction.

In summary, the co-immunoprecipitation conditions appear to remove all proteins besides the tagged STEVOR itself. Specifically, neither spectrin α nor actin could be confirmed as interacting partners. The lack of actin in the eluted fraction could possibly be explained by the poor staining observed in the western blot. Considering that the protein is barely detectable in the input fraction, it is possible that actin abundance dropped below detection levels in the elution. More striking was the presence of spectrin α in both positive as well as negative control. The previous report identifying spectrin α and β as interacting partner of Pf3D7_0631900 in gametocytes used episomally overexpressed protein with a C-terminal c-myc tag (Naissant et al., 2016). Pull downs were performed in four replicates using anti c-myc conjugated beads for co-immunoprecipitation. The negative controls were three replicates of co-immunoprecipitation from the same cell line with GST coupled beads as well as anti c-myc coupled beads with a null-expressing cell line. Replicates were averaged and an increase in uniquely detected peptides was shown. Furthermore, interaction was confirmed with recombinant C-terminal STEVOR domain and recombinant spectrin dimer.

While the data presented in the publication convincingly suggests interaction between the tagged STEVOR and spectrin α and β , the primary dataset provided in the supplementary material questions the validity. The four replicates suffer from tremendous variability (Spectrin α : 29, 93, 7, 2; Spectrin β : 35, 81, 6, 3; Ankyrin: 25, 66, 8, 10; Band 3: 24, 41, 4, 15), raising concerns about the average calculated from the replicates. Furthermore, the targeted STEVOR is only detected in two of the replicates with at least 2 unique peptides, a threshold commonly used as significance. Finally, the list of enriched hits has been heavily curated, excluding several proteins as "Plasma proteins" (P04114|APOB_HUMAN averages 26.25 vs 0.25 unique peptides in positive versus negative control to name the most strongly enriched example). While exclusion of proteins is often times an accepted option to improve the final

dataset obtained from co-immunoprecipitation, the study fails to acknowledge it entirely in the discussion and raises further questions about the interpretation of the co-immunoprecipitation. In light of these concerns, co-immunoprecipitation followed by mass spectrometric detection of interacting partners was performed despite the presence of spectrin α in the negative control.

3.5.3. STEVOR Pf3D7_0631900 and Pf3D7_1149900 only share two interacting proteins

Since the co-immunoprecipitation showed no contaminations from other proteins, the approach described was deemed suitable for mass spectrometric analysis. To this end, percoll enriched late stage parasites were lysed after DSP cross-linking with RIPA buffer. Lysates were split into two equal fractions and incubated with either anti-HA or anti-c-myc conjugated magnetic beads. Bound proteins were subjected to on-bead tryptic digest and peptides were detected by mass spectrometry. In order to differentiate between contaminant proteins and potential interacting partners, all proteins had to meet several criteria. Only proteins detected in all anti-HA bead replicates with at least two unique peptides per replicate were considered significant hits. To test for relative enrichment in the positive samples, both the unique and total peptide counts across all replicates were averaged. All proteins (excluding ribosomal proteins and keratin) with a ratio of at least 2:1 between positive and negative sample in either unique or total peptide count were considered potential hits (Table 5). Proteins are grouped depending on their presence in negative control replicates and sorted by their enrichment factor.

Pf3D7_0631900

Table 5: Table of proteins shortlisted as potential interaction partners of Pf3D7_0631900 and Pf3D7_1149900

Both STEVOR were detected in all positive samples but in none of the negative controls, in line with the preliminary western blot results. Surprisingly, only two proteins are considered potential interaction partners of both STEVOR: one uncharacterized Phistb (PF3D7_0424600) and HSP70-1 (PF3D7_0818900), a cytoplasmic parasite protein. HSP70-1 is one of several HSP70 proteins in the *P. falciparum* genome and has been associated with maintenance of proteostasis and stress response (Misra & Ramachandran, 2009; Shonhai, Botha, de Beer, Boshoff, & Blatch, 2008). The protein could potentially be involved in correct transport of STEVOR from the endoplasmic reticulum to the parasite plasma membrane, although there is no direct evidence to support this claim. No information is publicly available on the Phistb protein.

3.5.4. Pf3D7_0631900 is potentially anchored to the red blood cell membrane through interactions with Band 4.2

The list of potential Pf3D7_0631900 interaction partners includes several other parasite-internal or even nuclear localized proteins. Elongation factor 2 (eEF2, PF3D7_1451100), an essential factor in protein synthesis, shows a cytoplasmic localization (Baragana et al., 2015). Another molecular chaperone is HSP70-2 or BIP (PF3D7_0917900), which is considered endoplasmic reticulum resident (N. Kumar, Koski, Harada, Aikawa, & Zheng, 1991). Interestingly, it has been detected in a proteomic analysis of Maurer's Clefts and as a potential interacting partner of PfEMP1 (Baruch et al., 1996; Vincensini et al., 2005). Finally, T-complex protein 1 subunit alpha (TCP1, PF3D7_1132200) is a member of the TRiC complex. The chaperonin complex is located parasite internal and seems to play an important role in parasite biology and protein transport but is not essential for export out of the parasite (Spillman, Beck, Ganesan, Niles, & Goldberg, 2017). Among the nuclear proteins are histone H4 (PF3D7_1105000), Peroxiredoxin (PfnPrx, PF3D7_1027300, interacts with chromatin) and the GTP-binding nuclear protein (RAN, PF3D7_1117700, a Ras like protein which lacks any further characterization) (Dontfraid & Chakrabarti, 1994; Richard et al., 2011).

Among the confirmed exported proteins, Membrane associated histidine-rich protein (MAHRP2, PF3D7_1353200) is an essential component of the cylindrical structures that tether Maurer's Clefts to the red blood cell membrane (Pachlatko et al., 2010). Surprisingly, one A-type RIFIN (PF3D7_0115600) has been identified as potential interacting partner. No information is available for this particular protein. Considering that the samples for co-immunoprecipitation have been collected throughout several weeks, expression of this particular RIFIN appeared to remain stable. The remaining two proteins have been associated with the *P. falciparum* new permeability pathway. High molecular weight rhoptry protein 3 (RhopH3, PF3D7_0905400), which is part of the RhopH complex, is essential for parasite transition past the trophozoite stage (Cooper et al., 1988; Sherling et al., 2017). The only human protein in the list, Erythrocyte membrane protein band 4.2 (EPB42), interacts with the cytoskeletal proteins ankyrin and band 3 as well as with the before mentioned RhopH complex (Counihan et al., 2017; Korsgren & Cohen, 1988). Considering the identification of a RIFIN and the confirmed surface exposure of this particular STEVOR, the RhopH complex could potentially recruit sVSA in its vicinity in order to shield itself from immune recognition.

3.5.5. Pf3D7_1149900 interacts with lipid raft proteins

In contrast to the large number of parasite internal proteins identified in the Pf3D7_0631900 co-immunoprecipitation, Pf3D7_1149900 shows association with overall more exported proteins. The only parasite internal protein specific for this STEVOR is HSP90 (HSP86, PF3D7_0708400), an essential ATP dependant chaperone (Banumathy, Singh, Pavithra, & Tatu, 2003; Bonnefoy, Attal, Langsley, Tekaiia, & Mercereau-Puijalon, 1994).

Several of the identified proteins appear to be structural components interacting with the host cell cytoskeleton. One of the highest confidence hits in the co-immunoprecipitation was Skeleton-binding protein 1 (SBP1, PF3D7_0501300), a transmembrane protein in the Maurer's Clefts (Blisnick et al., 2000). The c-terminal domain of SBP-1, which is exposed into the red blood cell cytoplasm, interacts with protein 4.1R (band 4.1) and spectrin of the red blood cell cytoskeleton and is essential for correct localization of Maurer's Clefts (Cooke et al., 2006; Kats et al., 2015; Saridaki, Frohlich, Braun-Breton, &

Lanzer, 2009). Furthermore, SBP1 is essential for the transport of PfEMP1 from the Clefts to the red blood cell surface (Maier et al., 2007). The Mature parasite-infected erythrocyte surface antigen (MESA, PfEMP2, PF3D7_0500800) is structurally similar to PfEMP1 and interacts, like SBP1, with band 4.1 of the erythrocyte cytoskeleton (Coppel, 1992; Waller et al., 2003). Interestingly, wild-type parasites show a growth defect in the absence of band 4.1. In the absence of band 4.1 however, MESA knock-out parasites reverted back to normal growth as compared to the wild-type (Magowan et al., 1995). Erythrocyte membrane protein 3 (PfEMP3, PF3D7_0201900) is another parasite protein which interacts with the erythrocyte cytoskeleton through direct interaction with both spectrin as well as actin (Waller et al., 2007). Presence of PfEMP3 causes rigidification of the infected red blood cell as well as correct PfEMP1 presentation (Glenister, Coppel, Cowman, Mohandas, & Cooke, 2002; Waterkeyn et al., 2000).

Despite being one of the highest confidence candidates for interaction with Pf3D7_1149900, nothing is known about the Phistb protein PF3D7_0532300. The other Phistb protein (PfD80, PF3D7_0401800) specifically detected in the Pf3D7_1149900 co-immunoprecipitation has been identified in a proteomic analysis of Maurer's Clefts (Vincensini et al., 2005). The protein appears to locate to the red blood cell membrane, as GFP tagged protein is seen as a characteristic ring at the border of the cell (Tarr, Moon, Hardege, & Osborne, 2014). Besides identification in the same proteomic study of Maurer's Clefts, no information is available about PF3D7_0501000 (Vincensini et al., 2005).

Strikingly, several of the identified proteins have been previously associated with detergent resistant membranes, also termed lipid rafts. The human protein flotillin-1 is an integral membrane protein commonly used as marker for lipid rafts in red blood cells (Bickel et al., 1997; Salzer & Prohaska, 2001). Flotillins in general are ubiquitously expressed from bacteria to higher eukaryotes, and form membrane microdomains which are involved in, among others, signal transduction, clathrin-independent endocytosis and cell-cell adhesion (Frick et al., 2007; Glebov, Bright, & Nichols, 2006; Pandur, Dirksen, Moore, & Moody, 2004; Solis et al., 2007; Stuermer et al., 2001) (Reviewed in (Bodin, Planchon, Rios Morris, Comunale, & Gauthier-Rouviere, 2014)). The other human protein detected is

erythrocyte band 7 (stomatin, STOM), another integral membrane protein which is part of erythrocyte lipid rafts and a flotillin homologue (Hiebl-Dirschmied et al., 1991; Salzer & Prohaska, 2001; Tavernarakis, Driscoll, & Kyripides, 1999). It appears to be a scaffold protein and regulatory mechanism for several transporters and channel proteins (Price, Thompson, Eshcol, Wemmie, & Benson, 2004; Rungaldier, Oberwagner, Salzer, Csaszar, & Prohaska, 2013; Zhan et al., 2012). The high molecular weight rhoptry protein 2 (RhopH2, PF3D7_0929400) is, like the previously described RhopH3, part of the RhopH complex which forms the new permeability pathways in infected red blood cells (Cooper et al., 1988). It has been shown to directly interact with MESA as well as band 3, one of the channels interacting with band 7 (Counihan et al., 2017; Rungaldier et al., 2013). Furthermore, RhopH2 has been detected in detergent resistant membranes as well, suggesting association to lipid rafts (Hiller, Akompong, Morrow, Holder, & Haldar, 2003; Sanders et al., 2007). The putative endoplasmic GRP94 (HSP90 PF3D7_1222300) has been identified in proteomic analysis of trophozoite stage lipid rafts and subsequently described to localize to the endoplasmic reticulum (Yam et al., 2013). Finally, the Phistc protein PF3D7_0936800 directly interacts with the ATS domain of PfEMP1 underneath the erythrocyte membrane (Mayer, Slater, Erat, Konrat, & Vakonakis, 2012; Oberli et al., 2014). It has been detected in lipid rafts extracted from schizont staged parasites (Sanders et al., 2005).

In summary, the co-immunoprecipitation of two different STEVOR confirms previous theories about STEVOR processing and transport but also highlights the differences within the multigene family. Both proteins interact with several chaperones. Both interact with the parasite internal HSP70-1. While the surface exposed Pf3D7_0631900 shows interaction with BIP, the Maurer's Cleft resident Pf3D7_1149900 interacts with HSP90 as well as GRP94. It is unknown whether the difference in localization is the cause or effect for this observation. Both proteins show further interactions with different Phist proteins, many of which remain entirely unstudied. Considering that some of these proteins have been associated with PfEMP1 trafficking, it is likely that some Phist are involved in transport and anchorage of small variant surface antigens. One limiting factor in this analysis however is that Phist are multigene family as well, with parasite strains that express different subsets of Phist.

Therefore, no conclusion can be drawn whether knock-in of specific STEVORs causes expression of different subsets of Phist.

After arrival at their final destination, both proteins show a unique set of interacting partners. It appears that the surface exposed Pf3D7_0631900 is associated with the new permeability pathway which enables the parasite to access nutrients in blood plasma. Considering that the co-immunoprecipitation identified another small surface antigen, the RIFIN Pf3D7_0115600, it can be speculated that these proteins are inserted into the membrane to cover the essential components of the new permeability pathway against immune recognition. This is even further substantiated since no binding phenotype for Pf3D7_0631900 was identified so far. In this model, the pores created by the parasite proteins RhopH2/3 and Clag3.1 or Clag3.2 are surrounded by various members of small variant antigens which can easily be exchanged to other genes upon immune recognition. Furthermore, these antigens would most likely not be involved in host ligand binding since that could interfere with pore function. Proving this theory however would pose considerable difficulties as an absence of binding is almost impossible to unambiguously prove.

The Maurer's Cleft internal Pf3D7_1149900 on the other hand appears to be anchored in lipid rafts, where it interacts with a variety of transmembrane proteins. Interaction with well characterized Cleft resident proteins such as SBP1 as well as PfEMP2 and 3 confirms the hypothesis that the protein resides within the Maurer's Cleft. The strong interactions with lipid raft proteins is intriguing as it appears to be specific for this STEVOR. Interaction with the established lipid raft markers flotillin-1 as well as band 7 has to our knowledge not been reported for other parasite proteins before. Even though many of the identified proteins are involved in correct Cleft assembly and PfEMP1 transport, it can only be speculated what the function of this STEVOR is. Considering that it is far from essential for the parasite, it is unlikely that it functions as a structural protein or export chaperone. In rodent parasites, despite being even more numerous than in *P. falciparum* (*P. yoelii*: ~800 yir, *P. berghei*: ~100 bir and *P. chabaudi*: ~200 cir), function and even surface exposure of small variant antigens is largely unknown so far (Carlton et al., 2002; Cunningham, Lawton, Jarra, Preiser, & Langhorne, 2010; Hall et al., 2005; Janssen,

Barrett, Turner, & Phillips, 2002). It is debated what the function these multigene families could be, since antigenic variation seemingly requires surface exposure. According to the “smokescreen” theory the largest number of antigens presented to the immune system is present during schizont rupture (Cunningham et al., 2009). Variant antigens which are exported to the host cell but not exposed on the surface attract according to this theory the adaptive immune system at this timepoint. Considering that the protein is not present in merozoites but remains together with the red blood cell ghost and debris after schizont rupture, a “smokescreen” function is very appealing. It is of note however that the theory has been developed for rodent parasites which adapted, in contrast to *P. falciparum*, a hiding strategy within the red blood cell with few exported proteins (Sargeant et al., 2006). Very few proteins are presented on the surface of the infected red blood cell. *Plasmodium falciparum* on the other hand exports a large amount of proteins onto the host membrane and would therefore require the antigenic variation throughout the entire intra-erythrocytic part of the life cycle.

3.6. Identification of Pf3D7_1149900 within the Maurer’s Clefts through proximity biotinylation

In order to validate the potential interaction partners of Pf3D7_1149900 within the Maurer’s Clefts identified by co-immunoprecipitation, a proximity labelling approach was developed. BirA* is a promiscuous biotin ligase which constantly generates reactive biotin in its proximity (Roux, Kim, Raida, & Burke, 2012). Due to the highly reactive nature of the activated biotin, it will readily associate to primary amines in an effective labelling radius of approximately 10nm. This method is more suitable for the identification of large complexes considering that it labels not only directly interacting partners but also proteins in its vicinity as well as transient interacting partners. It has recently been used in a variety of studies in *P. falciparum* to identify proteins in the parasitophorous vacuole and its membrane (Khosh-Naucke et al., 2018; Schnider, Bausch-Fluck, Bruhlmann, Heussler, & Burda, 2018).

Mutagenesis of the ligase resulted in a more active protein, termed BioID2, which required both shorter labelling time as well as a reduced biotin concentration necessary in the culture medium (D. I.

Kim et al., 2016). Considering that malaria culture medium contains, in contrast to the medium used to culture mammalian cell in which BioID2 has been tested before, high levels of biotin (0.2mg/l compared to no biotin at all), the unmodified BirA* was chosen for further experiments (Dulbecco & Freeman, 1959; Moore, Gerner, & Franklin, 1967).

3.6.1. Tagging of Pf3D7_1149900 with the biotin ligase makes it the main *stevor* transcript

Pf3D7_1149900 was tagged with c-myc.BioID, analogous to the hemagglutinin tag constructs described before (Chapter 3.2.1). The c-myc tag provided a way for detection of the fusion protein. Quantitative RT-PCR for all 40 *stevor* was performed on the cell line, showing that once again the targeted protein became the strongest transcript (Figure 33, black arrow). This cell line however showed low levels of co-expression with another *stevor*, namely Pf3D7_1254300.

Figure 33: Quantitative real time PCR of all 40 *stevor* in the Pf3D7_1149900.c-myc.BioID knock-in cell line
 RNA of 1149900.c-myc.BioID was harvested 18 hours post invasion in triplicates and cDNA synthesized. Quantitative RT-PCR on all 40 annotated *stevor* in the 3D7 reference genome was performed and normalized to serine tRNA ligase. Relative mRNA was calculated. Error bars represent standard deviation. The targeted *stevor* was confirmed to be the main transcript. One other *stevor*, Pf3D7_1254300, showed co-expression in the cell line. Expression for all other *stevor* was either not detectable or at very low levels.

3.6.2. Tagging with BirA* does not alter protein localization

Localization of the fusion protein was confirmed by IFA (Figure 34). Even though the c-myc.BioID tag is considerably larger than the previously described hemagglutinin tag, localization of the protein appears to be similar between the two constructs (Figure 14c). Pf3D7_1149900.c-myc.BioID is exported beyond the boundaries of the parasite and appears to partially localize to sEMP1.

Figure 34: Localization of 1149900.c-myc.BioID in asexual stages by immunofluorescence assay
Parasites were fixed using methanol (trophozoite). Protein localization was determined by co-localization with EXP2 (PVM) or sEMP1 (Maurer's Cleft). DNA was stained with Hoechst33342. The fusion protein largely localizes to spots within the host red blood cell. These spots overlap with Maurer's Clefts. The staining pattern strongly mimics the previously described 1149900.3HA cell line

3.6.3. Pf3D7_1149900.c-myc.BioID is expressed at full length and functionally active

Expression of the fusion protein was controlled by western blot. Late stage parasites of the 1149900.c-myc.BioID and 3D7 strain were enriched on a percoll gradient and lysed in RIPA lysis buffer. The soluble fraction was run on a western blot and probed for presence of the c-myc tag (Figure 35a). A band of approximately the expected size can be detected in the transfected cell line but not in the 3D7 control, confirming expression of the full fusion protein.

Figure 35: Western blot analysis of BirA* tagged STEVOR

Pf3D7_1149900 was tagged with c-myc.BioID. (a) Late stage parasites of the transfected cell line and 3D7 were enriched on a percoll gradient and lysed in RIPA lysis buffer. Soluble fraction was run on a western blot and probed for c-myc. Band of the approximate size is only present in the transfected cell line (green arrow), confirming expression of the fusion protein. (b) Table indicating expected size of the fusion protein. (c) Transfected cell line and 3D7 were cultured with (+) or without (-) excess of biotin for 24hrs. Late stage parasites were harvested on a percoll gradient and lysed in RIPA buffer. The soluble fraction was run on a western blot and probed with fluorescently labelled streptavidin. 3D7 with or without biotin and 1149900.c-myc.BioID without biotin show a similar pattern of several faint bands. Upon incubation with excess biotin, the knock-in cell line displays several bands of varying intensity and molecular weight (red arrows). One band corresponds to the size of the fusion protein, as it was detected in the western blot analysis in panel a (purple arrow).

To confirm that the BirA* enzyme is fully functional within the Maurer's Clefts, the transfected cell line and a 3D7 control were incubated in excess biotin for 24hrs and harvested at late trophozoite stage by percoll gradient centrifugation. Cells were lysed in RIPA buffer and analysed by western blotting (Figure 35c). Fluorescently labelled streptavidin was used to detect biotinylated proteins. A band corresponding to the size detected in figure 35a can be seen only after incubation of the transfected

cell line in excess biotin (purple arrow), suggesting presence of the self-biotinylated protein. 3D7 parasites with and without biotin as well as the transfected cell line in absence of biotin share a few weak bands, indicating either naturally occurring biotinylated proteins within the infected red blood cell or unspecific binding of the probe. 1149900.c-myc.BioID with excess biotin however clearly displays several additional bands (red arrows), further confirming functionality of the biotin ligase.

3.6.4. Pf3D7_1149900.BioID proximity labelling identifies a wide array of parasite and host proteins

For mass spectrometric analysis, 1149900.c-myc.BioID and 3D7 were synchronized with sorbitol at 12 hours post invasion, incubated with excess of Biotin for 24 hours and finally harvested by percoll enrichment. For both cell lines, 5 samples were collected and lysed in RIPA buffer. Lysates were dialysed using Slide-A-Lyzer cassettes (2000 Dalton molecular cut-off) and incubated with streptavidin coupled Dynabeads. Beads were subjected to washes using 2% SDS, high salt RIPA buffer, 3M urea, RIPA and finally 100mM ammonium bicarbonate to wash away all unlabelled proteins. After mass spectrometric analysis, Pf3D7_1149900 could be detected in all 5 positive samples as a single unique peptide, but none of the 3D7 controls (data not shown). This confirmed on the one hand expression of the functional fusion protein, highlighted on the other hand that the bait protein itself was only detected in very low amounts. This could be either due to low starting abundance or over-washing of the beads.

The cut-off criteria applied in this pull down were very different from the previously described HA co-immunoprecipitation due to the inherent differences between the two methods. In this experiment, the harsh washes applied were supposed to remove all unspecific binding proteins from the beads. It was therefore assumed that every protein detected in the negative control is a contamination and not a true interaction partner. On the other hand, the very low peptide count of the bait protein suggested low abundance of interacting proteins as well. For a protein to be considered as potential interacting

partner, it had to be therefore detected in at least two of the positive samples. In summary, only proteins detected with two unique peptides in at least two positive samples and with no peptides detected in the no negative controls were considered as significant hit. Exceptions were made for two proteins: a putative ribonuclease (Pf3D7_0615400) and the knob-associated histidine-rich protein (KAHRP, Pf3D7_020200), both of which have been detected with two total and unique peptides in one negative control. Considering their high abundance in the positive samples, both proteins were included into the final list despite breaching the cut-off criteria.

elements	Protein Description	Total Peptides					avg	Unique Peptides					avg
Pf3D7_0615400	ribonuclease, putative	5	3	10	4	3	5	5	3	4	2	3	3
Pf3D7_0202000	knob-associated histidine-rich protein (KAHRP)	2	4	14	18	10	10	2	3	4	6	5	4
Pf3D7_0935900	ring-exported protein 1 (REX1)	10	14	6	22	7	12	9	10	3	11	7	8
Pf3D7_0730900	EMP1-trafficking protein (PTP4)	6	12	4	14	7	9	5	12	2	6	6	6
Pf3D7_0501200	parasite-infected erythrocyte surface protein (PIESP2)	2	2	4		2	3	2	2	2		2	2
Pf3D7_1016300	glycophorin binding protein (GBP)	3	7		12	2	6	3	4		5	2	4
Pf3D7_0402400	Plasmodium exported protein, unknown function (GEXP18)	2	3		8	2	4	2	3		4	2	3
Pf3D7_1002100	EMP1-trafficking protein (PTP5)	3	3		4		3	3	3		2		3
Pf3D7_0830500	product=tryptophan/threonine-rich antigen (TryThrA)	2	2		8		4	2	2		4		3
Pf3D7_1011800	PRE-binding protein (PREBP)		7		4	4	5		7		2	4	4
Pf3D7_0500800	mature parasite-infected erythrocyte surface antigen (MESA, PfEMP2)	3		8			6		3		4		4
Pf3D7_1136400	signal recognition particle subunit SRP72, putative (SRP72)		3		4		4		3		2		3
Pf3D7_0501300	skeleton-binding protein 1 (SBP1)		2			2	2		2			2	2
Pf3D7_0710200	conserved Plasmodium protein, unknown function		3			2	3		3			2	3
Pf3D7_0922200	S-adenosylmethionine synthetase (SAMS)		2			2	2		2			2	2
Pf3D7_0929400	high molecular weight rhoptry protein 2 (RhopH2)		2			2	2		2			2	2
Pf3D7_1246200	actin I (ACT1)		2				3		2			2	2
Pf3D7_0708400	heat shock protein 90 (HSP90)		4		6		5		4		3		4
Pf3D7_0818900	heat shock protein 70 (HSP70)		3		4	2	3		3		2	2	2
Pf3D7_0831700	heat shock protein 70 (HSP70-x)		2		4		3		2		2		2
Pf3D7_0917900	heat shock protein 70 (HSP70-2, BIP)		5		12	2	6		5		5	2	4
Pf3D7_1222300	endoplasmic, putative (GRP94, HSP90)		4			2	3		4			2	3
Pf3D7_0831700 / Pf3D7_0818900	heat shock protein 70 (HSP70-x) / heat shock protein 70 (HSP70)		2			2	2		2			2	2
Pf3D7_0317600	40S ribosomal protein S11, putative (RPS11)	2	2				2	2	2				2
Pf3D7_0322900	40S ribosomal protein S3A, putative		2		4		3		2		2		2
Pf3D7_0710600	60S ribosomal protein L34 (RPL34)	2	2	6			3	2	2	3			2
Pf3D7_0821700	60S ribosomal protein L22, putative		2		4		3		2		2		2
SLC4A1	Band 3 anion transport protein	2	3	4	4		3	2	3	2	2		2
CASP14	Caspase-14	3	2				3	3	2				3
LGALS7	Galectin-7	2	2				2	2	2				2
FABP5	Fatty acid-binding protein, epidermal	3	2				3	3	2				3
EPX	Eosinophil peroxidase	2		8			5	2		4			3
KRT14 / KRT13 / 17 / 16	Keratin, type I cytoskeletal 14 / Keratin, type I cytoskeletal 13 / Keratin, type I cytoskeletal 17 / Keratin, type I cytoskeletal 16	2			4		3	2			2		2
HBD / HBB	Hemoglobin subunit delta / Hemoglobin subunit beta	3	3		4		3	3	3		2		3

Table 6: List of proteins in the vicinity of the Pf3D7_1149900 C-terminus, identified through proximity labelling by BirA*

The list of proteins identified after application of the cut-off criteria can be seen in table 6. The next chapter will discuss the proteins in more detail.

3.6.5. Proximity labelling confirms some, but not all proteins identified through co-immunoprecipitation

The list of proteins identified through proximity labelling partially overlaps with the potential interaction partners suggested from the co-immunoprecipitation of 1149900.3HA. The list of proteins includes SBP1 (Pf3D7_0501300), MESA (Pf3D7_500800, PfEMP2) and RhopH2 (Pf3D7_0929400). Both SBP1 as well as MESA interact with Band 4.1 of the erythrocyte cytoskeleton. RhopH2 on the other hand is interacting with MESA and Band 3. Band 3 is an anion channel in the red blood cell membrane and has been identified through proximity labelling as well. Considering the mutual interactions previously described for these proteins, as well as their identification through two entirely independent methods, the STEVOR Pf3D7_1149900 could potentially be part of a structural complex which anchors the Maurer's Clefts to the red blood cell membrane.

Further proteins identified through both proximity labelling as well as co-immunoprecipitation are the heat shock proteins Pf3D7_0708400 (HSP90, HSP86), Pf3D7_1222300 (GRP94, HSP90) and Pf3D7_818900 (HSP70-1). It is of note that these proteins are parasite internal, suggesting biotin labelling early after translation and before the protein has been exported into the host red blood cell. The identification of 4 different ribosomal proteins (Pf3D7_0317600, Pf3D7_0322900, Pf3D7_071600 and Pf3D7_0821700) further suggests activity of the ligase early after translation.

Comparing the two lists of interaction partners, there is a striking lack of Phist proteins in the list identified by proximity labelling. According to co-immunoprecipitation, Pf3D7_1149900 interacts with 4 proteins of this multigene family (Pf3D7_0532300, Pf3D7_0401800, Pf3D7_0936800 and Pf3D7_0424600). Considering that the proximity labelling approach will only identify proteins on the within the Maurer's Clefts but not in the host cell cytosol, these proteins might potentially never be exposed to the BirA* enzyme.

3.6.6. C-terminal tagging of STEVOR with BirA* causes labelling of Maurer's Clefts resident proteins

In addition to the previously mentioned Maurer's Cleft resident SBP1, MESA and RhopH2, proximity labelling identified several other proteins that have been shown to reside within this compartment or are trafficked through it.

The ring-exported protein 1 (Pf3D7_0935900, REX1) is resident and involved both in Maurer's Cleft stabilization as well as PfEMP1 export (Dixon et al., 2011; Hanssen, Hawthorne, et al., 2008; Heiber et al., 2013). Knockout of the two EMP1-trafficking proteins PTP4 and PTP5 (Pf3D7_0730900 and Pf3D7_1002100 respectively) disrupted PfEMP1 export and cytoadhesion of the infected red blood cells (Maier et al., 2008). Due to the phenotypic similarity to PF3D7_0202200, it is suggested that both proteins localize to Maurer's Clefts as well as the host cell cytosol. GFP-tagged TryThrA (Pf3D7_0830500) partially localizes to Clefts from trophozoite stage onwards (Heiber et al., 2013). The remaining protein remains within the endoplasmic reticulum. Peptides spanning several regions of the protein block merozoite invasion (Curtidor et al., 2006).

Among the proteins trafficked through the Clefts is the knob-associated histidine-rich protein (KAHRP, Pf3D7_020200), which has been shown to be trafficked through the Clefts on its way to the erythrocyte membrane (Wickham et al., 2001). The parasite-infected erythrocyte surface protein (PIESP2, Pf3D7_0501200) can be found both in the host cell membrane as well as Maurer's Clefts (Florens et al., 2004; Vincensini et al., 2005).

So far, the orientation of Pf3D7_1149900 in the membrane of Maurer's clefts was so far not proven. Considering the identification of numerous Maurer's Clefts resident or trafficked proteins, it is likely that the C-terminus of the protein is located within the Cleft while the N-terminus is in the host cell cytosol. A further argument for this orientation is the lack of the two previously identified host cell proteins, Flotillin-1 and Band 7.

3.6.7. Non-Cleft resident proteins are possibly involved in STEVOR transport

One of the non-Cleft resident proteins identified, which is neither a chaperone nor part of the ribosome, is the glycophorin binding protein (GBP130, Pf3D7_1016300). The exported protein remains in the host cell cytosol, where it proposed to bind to glycophorin (Kochan, Perkins, & Ravetch, 1986; Ravetch, Kochan, & Perkins, 1985). This binding has been questioned later on (van Schravendijk, Wilson, & Newbold, 1987). The Plasmodium protein of unknown function (GEXP18, Pf3D7_0402400) has been identified in proteomic studies of J-Dots, together with HSP70-x (Pf3D7_0831700) (Q. Zhang et al., 2017). The protein complex containing HSP70-x and GEXP18 interacts with parts of GBP130. Since J-Dots have been previously associated with STEVOR export, it is likely that all three of these proteins were labelled during the transport of the fusion protein to the Maurer's Cleft (Rug et al., 2014).

Despite the stringent cut-off applied for the identified proteins, several of the shortlisted candidates can most likely be considered contaminants. The PRE-binding protein (PREBP, Pf3D7_1011800) is a nuclear transcription factor (Komaki-Yasuda, Okuwaki, Nagata, Kawazu, & Kano, 2013). The putative protein Pf3D7_0615400 is predicted to be ribonuclease, an RNA degrading enzyme. SRP72 (Pf3D7_1136400) is a putative member of the signal recognition particle. Signal recognition particles are a family of proteins that targets proteins to the endoplasmic reticulum in eukaryotes (Walter, Ibrahimi, & Blobel, 1981). The S-adenosylmethionine synthetase (SAMS, Pf3D7_0922200) has been linked to maintenance of cellular redox homeostasis (Pretzel et al., 2016). Finally, ACT1 (Pf3D7_1246200) is essential for apicoplast segregation and merozoite development (Das, Lemgruber, Tay, Baum, & Meissner, 2017). No information is publicly available on the conserved protein Pf3D7_0710200, despite it being predicted to be essential (M. Zhang, Wang, et al., 2018).

In summary, proximity labelling of Pf3D7_1149900 interacting proteins identified a wide range of proteins, partially overlapping with the co-immunoprecipitation results and further confirming mode of STEVOR export.

Identification of HSP70, GEXP18 and GBP130 strongly suggests transport of STEVOR by J-Dots from the parasitophorous membrane to the Maurer's Clefts. It is of note that the biotin ligase appears to be

active immediately after translation. This helps with the identification of export factors as well as parasite chaperones on the one hand, introduces a large amount of parasite internal proteins as contaminations on the other hand such as the four ribosomal proteins identified.

Within the Maurer's Clefts, proximity labelling confirmed some of the interactions identified through co-immunoprecipitation. SBP1, PfEMP2 as well as RhopH2 have been associated with Clefts before. Considering that co-immunoprecipitation and proximity labelling are entirely independent approaches to identify protein - protein interactions, overlapping hits are strong candidates for true interactions. Even though the proposed effective labelling distance of 10nm is very short, it has to be kept in mind that BioID method identifies not only interacting but also adjacent proteins. The long list of additional Maurer's Cleft proteins identified by BioID is a testament to that. On the other hand, it did not identify the human proteins flotillin-1 and band 7. Either the distance from the ligase to the human protein is so far that labelling is not probable enough anymore, or STEVOR interaction happens at the N-terminal, cytoplasmic part of the protein. The potential role of Pf3D7_1149900 in the Cleft has been discussed in the previous chapter, raising the possibility of a "smokescreen" during merozoite rupture. Even though the BioID proximity labelling confirmed the co-immunoprecipitation to a large degree, it did not offer a different, more active role of the parasite other than immune evasion.

3.7. Knock-in of immune suppressive rif causes resistance to NK cell killing

Both proteins have been described to interact with receptors on the surface of immune cells. To confirm functionality of the tagged fusion protein, natural killer cell killing assays were performed (Figure 36, performed by Marvin Chew, unpublished data). Parental 3D7 ("3D7 WT") as well as the *var* knock-in cell line ("VAR") were used as negative control. The PfEMP1 knock-in cell line served as control to exclude any effect of GFP or the SLI system on the assay.

Both RIFIN knock-in cell lines (1101100.GFP termed "RIFIN 11" and 1254800.GFP termed "RIFIN 12") cell lines show significant decrease in natural killer cell mediated clearance compared to both negative controls, proving that knock in of these RIFINs retains their functionality.

Figure 36: Natural Killer cell killing assay on parental 3D7, the two RIFIN and the PfEMP1 knock-in cell lines

Natural killer cell killing assay was performed by co-culturing of NK-cells with parasitized blood for 48hours. Final parasitemia was assessed by flow cytometry and compared to the growth of parasites without NK-cells. Both 1101100.GFP as well as 1254800.GFP (RIFIN 11 and RIFIN 12 respectively) are significantly less susceptible to NK-cell clearance than 3D7 or 0421300.GFP (3D7 WT and VAR). Experiment was performed by Marvin Chew, unpublished data.

Both are knocked in *rif* are considered A-type RIFIN and have been previously associated with suppression of the innate immune system (Saito et al., 2017). Direct binding of Pf3D7_1254800 to its target receptor was shown, causing inhibition of Natural killer cells. The second *rif*, Pf3D7_1101100, triggered activation of a downstream reporter system, even though evidence of direct binding was lacking (Saito et al., 2017). Strikingly, only Pf3D7_1101100 was exported into the host red blood cell, while Pf3D7_1254800 remained parasite internal. According to previous data, all A-type RIFIN are predicted to be exported. Our findings clearly contradict this theory and provide evidence that the established grouping of RIFIN is not fully accurate (Bultrini et al., 2009; Goel et al., 2015). Both cell lines were resistant to natural killer cell killing, confirming their previously described function. Considering that Pf3D7_1254800 is not exported, interaction with host immune cells must be achieved through other means than direct cell-cell interaction. It has been previously described that *P. falciparum* is producing exosomes and microvesicles capable of communication with other parasites, red blood cells and endothelial cells (Mantel et al., 2016; Mantel et al., 2013; Regev-Rudzki et al., 2013). Moreover,

they can harbour RIFINs in clinical isolates, a situation when immune suppression is of utmost importance for the parasite (Abdi et al., 2017). The original publication characterizing the two RIFINs utilized a mammalian cell surface expression to show interaction with their target receptors on the surface of immune cells (Saito et al., 2017). Considering that both RIFIN are present in merozoites, interaction with immune cells could be mediated during the extra erythrocytic part of the life cycle. It has been shown that antibodies against STEVOR can inhibit invasion at an early stage, showing that the protein is presented on the surface of the merozoite at its apical tip (Niang et al., 2014). The RIFIN 1254800.GFP appears to accumulate at a distinct spot, possibly presenting the protein on the surface. It could be imagined that RIFIN that are presented on the surface of merozoites interact with immune cells, thus sacrificing some merozoites in order to suppress immune cells and aid the remaining parasites. Either way, the interaction between RIFIN and its target receptor appears to be more complicated than expected and is far from being fully understood.

4. Summary and Conclusions

This work provides novel insights into the biology surrounding the *stevor* multigene family and a methodology to study members of multigene families in their native environment.

Numerous aspects of STEVOR biology have been investigated in this work. Using recombinant protein, a protein with novel binding properties to uninfected red blood cells was identified. This particular gene falls into a group which shows high divergence from most other *stevor*. Considering that both *rif* as well as *var* show interaction with several host cell ligands, it is not surprising to identify additional receptors utilized by *stevor*. Nonetheless, this finding provides to our knowledge the first indication of STEVOR binding to a receptor other than Glycophorin C.

In order to study variant surface antigens in a more comprehensive and rationale driven way than before, this work describes an approach to force expression of any member of multigene families by *P. falciparum*. By tagging of the genomic locus with the recently published SLI method, parasites are positively selected for integration of the construct into its genome as well as active expression of the fusion protein. To validate this approach, one *var*, two *rif* and three *stevor* were tagged and validated for correct expression. It could be shown that the so far unstudied Pf3D7_0421300 is presented on the surface of the infected red blood cells, as is expected from a PfEMP1 protein.

Forcing the parasite to express either of the two *rif* Pf3D7_1101100 or Pf3D7_1254800 makes the parasite resistant to natural killer cell mediated killing. This confirms the role of this multigene family in immune suppression. Furthermore, Pf3D7_1254800 is apparently not exported into the host red blood cell but remains within the parasite. This surprising finding not only suggests that the classification of RIFIN into A and B type is insufficient, it also proposes a more elaborate mode of ligand interaction than presentation on the surface of infected red blood cells. It can be speculated that the protein is transported via microvesicles or that it is presented on the surface of merozoite, even though experimental validation of these theories is missing at this point in time.

The three STEVOR investigated highlight the diversity within the family that has been described before. While Pf3D7_0631900 is presented on the surface of the infected red blood cell, Pf3D7_1149900 remains within the Maurer's Clefts. Pf3D7_0102100 is lacking a start codon according to Plasmo_DB and most likely utilizes an alternative one, as it is expressed in a truncated form. Complement DNA apparently does not start from the predicted start site, further suggesting a gene internal start site instead of alternative splicing.

Not only do Pf3D7_0631900 and Pf3D7_1149900 show entirely different localizations, both proteins co-immunoprecipitate with a unique set of parasite and host proteins, indicating different roles of the proteins in immune evasion. The surface exposed Pf3D7_0631900 interacts with Band 4.2 and RhopH3, both of which are components of the new permeability pathway, porous complexes inserted into the host membrane to improve nutrient uptake for the parasite. Together with another identified RIFIN, it can be imagined that the essential components of those pores are shielded from the immune system by small variant antigens, thus making the parasite more resistant to immune recognition. Furthermore, interaction with the Maurer's Cleft tether MAHRP2 indicates anchorage of the STEVOR to these tethers rather than the host cell cytoskeleton.

The Maurer's Cleft internal Pf3D7_1149900 on the other hand is apparently anchored in lipid rafts within the Cleft, as is suggested by the interaction with the well-established human lipid raft proteins flotillin-1 and Band 7. Residence within in the Cleft is shown by interaction with SBP1, PfEMP2 and 3. These proteins are known to either reside in or are transported through Maurer's Clefts. Furthermore, these interactions have been confirmed through proximity ligation using BioID. Considering that neither flotillin-1 nor Band 7 are identified by BioID, it can be assumed that they interact with the N-terminal part of the STEVOR. Since structural roles of a non-essential protein are unlikely, we hypothesize that the protein serves as a smokescreen during schizont rupture, a theory postulated for rodent antigens previously.

Finally, knock-in of members of the *stevor* or *rifin* multigene families gave valuable insights into the regulatory mechanisms within the families. It is well established that *var* are under strict mutual exclusive control but little information is available for the small variant antigens. Here we could show that knock-in of a *stevor* causes downregulation of the entire rest of the family, an observation indicating exclusive expression. This could not be seen for *rif*, which are expressed in large numbers even after knock-in of one. Furthermore, we could find indications of cross-talk between the different multigene families. Unspecific cross-talk has been already associated with an un-identified “titrable factor” which caused downregulation of entire families. Here we show expression from a specific locus causes upregulation of adjacent genes over several kilo base pair distances. This observation, even though it has to our knowledge not been voiced before, could be seen in other published studies of *P. falciparum* clones as well, suggesting that it is not an arte fact of the SLI method but a true phenomenon.

5. Future work

The work presented here can be loosely grouped into the establishment of the knock-in method and studies surrounding STEVOR biology. At this point in time, we consider the method development finished and established. Therefore, the loose ends and future projects revolve around functional analysis of the proteins that were knocked in. Since the *stevor* multigene family is in the focus of this work, most future projects involve this multigene family.

- Creation of a STEVOR knock-in library

To this day, a comprehensive study of STEVOR is lacking. The studies describing certain members either express them in other systems, use episomal expression in *P. falciparum*, clonal lines and / or specific antibodies (Bachmann et al., 2012; Bachmann et al., 2015; Blythe et al., 2008; Niang et al., 2014; Saito et al., 2017; Wichers et al., 2019). All of these approaches are prone to errors in one way or another. Expression in other systems does not guarantee correct folding and post-translational modifications. This is apparent in the differences in membrane topology observed (Wichers et al., 2019). Episomal expression disrupts temporal specificity, possibly creating artefacts depending on peak expression. Clonal lines only express *stevor* by chance and therefore do not allow to choose which genes to study. Finally, raising antibodies against a single variant antigen causes difficulties due to their similarity and is even more troublesome to prove specificity. We therefore propose the generation of a library, covering at least 15 genes out of the 40 which are present in 3D7 parasites. This library would cover a broad diversity of genes, thus enabling to study the full functional diversity present in the family. Parasites expressing specific STEVOR can be screened for rosetting, sequestration and immune suppression.

- Further validation of intracellular STEVOR interaction partners

This work presents for the first time a list of proteins interacting with STEVOR at different locations. Even though the large number of replicates gives a lot of confidence in the co-immunoprecipitation results and interacting partners of Pf3D7_1149900 have been partially confirmed by proximity ligation, they require validation. The first step is co-immunoprecipitation of interacting partners in the

appropriate knock-in cell line. Furthermore, lipid raft association of Pf3D7_1149900 can be validated by lipid raft isolation (Yam et al., 2013). Lipid raft disruption has been previously shown to inhibit PfEMP1 export (Frankland et al., 2006). It is unclear so far whether lipid rafts are essential for STEVOR surface exposure as well. It would therefore be worthwhile to study the effect of cholesterol depletion using methyl-beta-cyclodextrin on Pf3D7_0631900 surface exposure. Furthermore, destabilization of lipid rafts followed by immune-precipitation can be utilized to show direct binding between Pf3D7_1149900 and flotillin as opposed to unspecific interaction in the protein dense lipid raft environment. The human proteins identified by co-immunoprecipitation can be further validated in lab-grown red blood cells (Kanjee et al., 2017). Invasion of red blood cells derived from pre-cursor cells by *P. falciparum* has recently been established. Knock-out or -down of human proteins using CRISPR/Cas9 can therefore be used to study the importance of those proteins on correct STEVOR localization.

Finally, phosphorylation status of STEVOR appears to be important for correct localization (Naissant et al., 2016). Introduction of point mutations into the C-terminus of STEVOR can inhibit phosphorylation by PKA. While this changes stiffness of gametocytes, nothing is known about the impact in asexual stages. Co-immunoprecipitation of phosphorylation deficient STEVOR can answer whether phosphorylation is essential for interaction with certain proteins or even surface exposure of the protein.

Epigenetic factors involved in *stevor* expression

So far, nothing is known about the epigenetic regulation within the *stevor* family. A previous PhD student from our lab, Tatenda Mushunje, studied the histone modifications present at active and inactive loci. To this end, he performed chromatin immune-precipitation on different parasite clones with unique *stevor* expression patterns. Even though this approach has been utilized before to study *var* gene expression, it compares to independent cell lines, potentially introducing artefacts (Chookajorn et al., 2007; Lopez-Rubio et al., 2007). By studying histone occupancy and modifications

in the 1149900.3HA “off” clone and its re-activated counterpart, clonal differences can be excluded. Furthermore, the same cell lines can be used to study nuclear localization of the *stevor* locus. It is unknown so far whether relocation of the active locus from the nuclear periphery to an “active cluster” is *var* specific or common for all variant antigens (Dzikowski et al., 2007; Freitas-Junior et al., 2000).

- Investigate the mode of *rif* Pf3D7_1254800 immune suppression

The final project emerging from this work is to investigate how the RIFIN Pf3D7_1254800 interacts with natural killer cells. Considering that it is not exposed on the surface of infected red blood cells, we hypothesize that it is either on the surface of merozoites or exported in vesicles.

In order to investigate merozoite presentation of the protein, antibodies broadly raised against the N-terminal domain of RIFIN should detect the protein in live merozoites. Several antibodies against different subsets of RIFIN have been characterized before, providing an excellent opportunity to study Merozoite exposure (Ch'ng et al., 2017). Extracellular vesicles from harbouring RIFIN have been described before (Abdi et al., 2017). Furthermore, isolation of vesicles from culture supernatant is well established (Mantel et al., 2013). Either way, interaction of the RIFIN with immune cells through other means than exposure on the surface of red blood cells highlights the depth of techniques utilized by parasite to interact with host cells.

6. References

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