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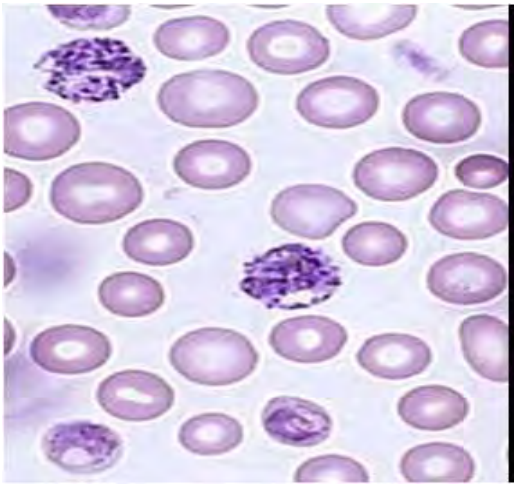

Μανακανάτα Χριστίνα

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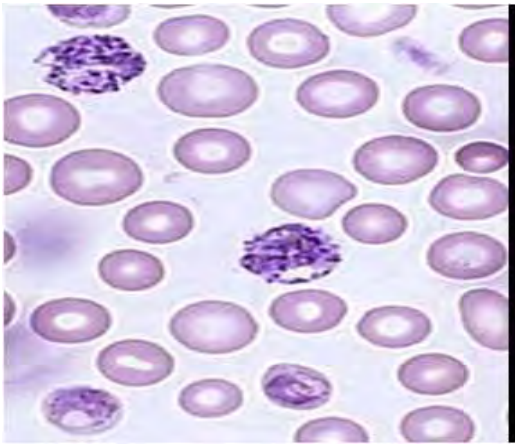
Development of Tetracycline Repressor Aptamer system in *Plasmodium berghei*



Manakanata Christina

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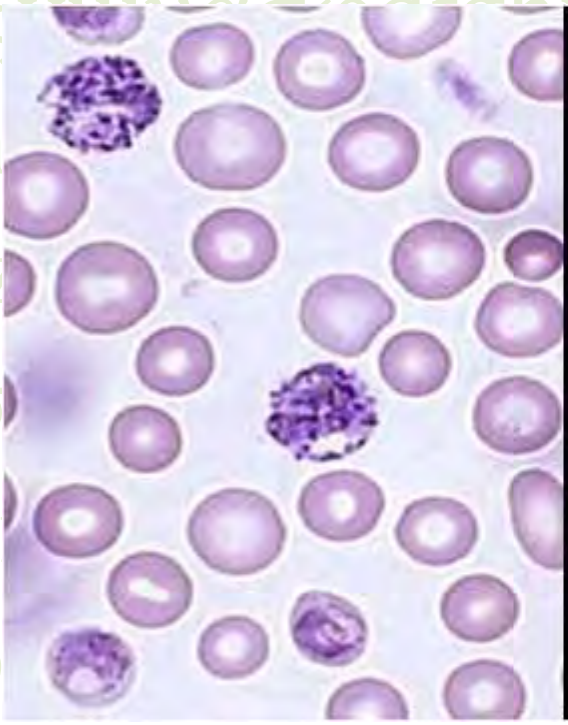
10/6/2014

Christina Manakanata



Ανάπτυξη του συστήματος καταστολέα τετρακυκλίνης-απταμερούς στο Plasmodium berghei

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Development of Tetracycline Repressor Aptamer system in Plasmodium berghei

Τριμελής επιτροπή:

Μαθιόπουλος Κώνσταντίνος

Τσουμάνη Κωνσταντίνα

Andrew P. Waters

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1.Περίληψη

Η ελονοσία είναι μια μολυσματική ασθένεια που μεταδίδεται από το κουνούπι και μπορεί να προσβάλλει τον άνθρωπο και άλλα ζώα. Αυτή η ασθένεια είχε πάντοτε πολύ σοβαρές συνέπειες για την ανθρωπότητα, δεδομένου του ότι είναι δύσκολο να ελεγχθεί η επέκτασή της και ως εκ τούτου, η ελονοσία μπορεί εύκολα να γίνει μια ευρέως διαδεδομένη ασθένεια όπως η ιστορία έχει δείξει τόσες φορές. Ο αιτιολογικός παράγων της ελονοσίας είναι ένα παρασιτικό πρωτόζωο που ονομάζεται *Plasmodium* και ανήκει στην συνομοταξία *Apicomplexa*, ενώ φορέας μετάδοσης της είναι το θηλυκό κουνούπι *Anopheles*. Αυτό το ασπόνδυλο μεταδίδει σποροζωίδια, τα οποία είναι μια κινητική μολυσματική μορφή του *Plasmodium*, στον σπονδυλωτό ξενιστή κατά το δάγκωμα. Τα σποροζωϊδια ταξιδεύουν προς το ήπαρ μέσω των αιμοφόρων αγγείων και εκεί μολύνουν τα ηπατοκύτταρα. Εκεί αναπαράγονται αγενώς και παράγουν χιλιάδες μεροζωΐτες, τα οποία έχουν τη δυνατότητα να μολύνουν τα ερυθρά αιμοσφαίρια, όπου το παράσιτο ξεκινά μια σειρά από αγενή αναπαραγωγικούς κύκλους με αποτέλεσμα να παραχθούν νέα μεροζωίδια. Μερικά μεροζωϊδια έχουν τη δυνατότητα να αναπτυχθούν σε ανώριμα γαμετοκύτταρα και ακολούθως να σχηματίσουν γαμέτες, αφού προσληφθούν από ένα νέο κουνούπι κατά τη διάρκεια ενός γεύματος. Μέσα στο στομάχι αυτού του κουνουπιού ο αρσενικός και θηλυκός γαμέτης ενώνονται και σχηματίζουν έτσι έναν οοκινέτη, ο οποίος είναι η κινητή μορφή του ζυγώτη. Οι οοκινέτες αναπτύσσονται και σχηματίζουν νέα σποροζωϊδια τα οποία μεταναστεύουν στους σιελογόνους αδένες και έτσι ο κύκλος ζωής ξεκινάει από την αρχή.

Το *Plasmodium falciparum (P. falciparum)* είναι το ένα από τα τέσσερα γνωστά παράσιτα υπεύθυνα για την πρόκληση ανθρώπινης ελονοσίας το οποίο είναι υπεύθυνο για τις περισσότερες περιπτώσεις λοιμογόνους ελονοσίας παγκοσμίως. Ως εκ τούτου, η μελέτη του γονιδιώματος του θα μπορούσε να οδηγήσει σε νέες προσεγγίσεις για την ανάπτυξη εμβολίων για την αποτελεσματική αντιμετώπιση της ελονοσίας. Παρ 'όλα αυτά, συχνά οι ερευνητές χρησιμοποιούν το *Plasmodium berghei*, ένα παράσιτο υπεύθυνο για πρόκληση ελονοσίας στα τρωκτικά, αντί για το *P. falciparum* επειδή το *P. berghei* είναι μη λοιμώδες για τον άνθρωπο, ενώ η οργάνωση του γονιδιώματος και η γενετική μεταξύ αυτών των δύο ειδών είναι εξαιρετικά συντηρημένη.

Τα παράσιτα του γένους *Plasmodium* είναι απλοειδή καθ’ όλη τη διάρκεια ζωής τους, εκτός από τα στάδια που μεσολαβούν από το ζυγώτη ως την ωοκύστη, και ως εκ τούτου η εξάλειψη τυχόν απαραίτητων γονιδίων θα μπορούσε να προκαλέσει θανατηφόρο φαινότυπο. Για αυτόν τον λόγο η ανάπτυξη τεχνικών κατά συνθήκη knockdown-out προκειμένου να μελετηθούν αυτά τα απαραίτητα γονίδια απαιτείται. Η παρούσα έρευνα αποσκοπεί στην ανάπτυξη του συστήματος του καταστολέα τετρακυκλίνης- Απταμερούς για το *Plasmodium berghei.* Το σημαντικό πλεονέκτημα αυτού του συστήματος είναι ότι τα επίπεδα πρωτεΐνης μπορούν να ρυθμιστούν αναλόγως με την συγκέντρωση τετρακυκλίνης που παρέχεται. Τα απταμερή είναι μοναδικές αλληλουχίες που μπορούν να δεσμεύσουν μικρά μόρια και RNA δομές (καταστολέας) και έτσι να παρεμποδίσουν την έκφραση του γονιδίου του οποίου προηγούνται. Κατά την απουσία τετρακυκλίνης ο καταστολέας μπορεί

να προσδεθεί στο απταμερές αποτελεσματικά και έτσι η έκφραση του γονιδίου καταστέλλεται. Όταν η τετρακυκλίνη παρέχεται ο καταστολέας προσδένεται στο μόριο αυτό και όχι στο απταμερές, επιτρέποντας έτσι την έκφραση του γονιδίου.

Για την ανάπτυξη του συστήματος του καταστολέα τετρακυκλίνης-απταμερούς στο *P.berghei* διάφορες διαγονιδιακές σειρές ελέγχου αναπτύχθηκαν επιτυχώς. Πρώτα, δύο διαγονιδιακές σειρές που εκφράζουν τον καταστολέα της τετρακυκλίνης υπό τον έλεγχο είτε του HSP70 (συστατική έκφραση του καταστολέα τετρακυκλίνης) ή του P25 (έκφραση του καταστολέα μόνο μετά την ενεργοποίηση των γαμετοκυττάρων) προαγωγέα παρήχθησαν και ή έκφραση του καταστολέα επιβεβαιώθηκε με Western blotting και για τις δύο σειρές. Περαιτέρω, η σειρά που εκφράζει τον καταστολέα συστατικά επιλέχθηκε θετικά και κλωνοποιήθηκε. Αυτή η κλωνοποιημένη σειρά στη συνέχεια ελέγχθηκε πάλι με Western blotting για να επιβεβαιωθεί η έκφραση του καταστολέα και εξετάστηκε για τυχόν ελαττώματα στην ανάπτυξη των παρασίτων. Επόμενο βήμα ήταν να επιλεχθεί αρνητικά έτσι ώστε να χρησιμοποιηθεί για περαιτέρω επιμολύνσεις. Προκειμένου να δοκιμαστεί η επίδραση ενός λειτουργικού και ενός μη λειτουργικού απταμερούς στην έκφραση του γονιδίου αναφοράς (*gfp*) δύο διαγονιδιακές σειρές παρασίτων που εκφράζουν είτε τον λειτουργικό είτε το μη λειτουργικό απταμερές μπροστά από την αλληλουχία *gfp* χρειάστηκε να αναπτυχθούν. Μόνο η διαγονιδιακή σειρά που εκφράζει την αλληλουχία του μη λειτουργικού απταμερούς-gfp δημιουργήθηκε με επιτυχία, ενώ για τη δημιουργία της σειράς που έπρεπε να εκφράζει την αλληλουχία του λειτουργικού απταμερούς-*gfp* όλες οι προσπάθειες δημιουργίας ενός φορέα επιμόλυνσης (πλασμίδιου) κατέληξαν σε ένα μεταλλαγμένο μοτίβο λειτουργικού απταμερούς-*gfp*. Περισσότερες προσπάθειες για την ανάπτυξη αυτής της διαγονιδιακής σειράς πρέπει να διεξαχθούν. Τελικώς, μια διαγονιδιακή σειρά που να χρησιμεύει ως θετικό control εκφράζοντας μόνο την *gfp* αλληλουχία αναπτύχθηκε με επιτυχία. Ως αρνητικό control θα χρησιμοποιηθεί μια wild type *HPTBB* σειρά παρασίτων. Όλες οι διαγονιδιακές σειρές παρασίτων παρήχθησαν χρησιμοποιώντας πλασμίδια ως φορείς επιμόλυνσης και η επιμόλυνση πραγματοποιήθηκε με ένα γεγονός διπλού ομόλογου ανασυνδιασμού.

Μελλοντικές προοπτικές για το σύστημα καταστολέα τετρακυκλίνης-Απταμερούς περιλαμβάνουν την επιμόλυνση των φορέων που εκφράζουν την αλληλουχία *gfp:λειτ/μη λειτ.απταμερές* στις διαγονιδιακές σειρές που εκφράζουν τον καταστολέα τετρακυκλίνης έτσι ώστε να προκύψουν οι τέσσερις τελικές σειρές παρασίτων που θα χρησιμοποιηθούν για τον έλεγχο της λειτουργικότητας του συστήματος. Μετά την κλωνοποίηση αυτών των σειρών και τον έλεγχο τους για τυχόν ελαττώματα ανάπτυξης θα χρησιμοποιηθούν για τη δοκιμή του συστήματος παρουσία και απουσία της τετρακυκλίνης. Επίσης, η αναστρεψιμότητα του συστήματος με την προσθήκη της τετρακυκλίνης, καθώς και η δυνατότητα να κατασταλεί εντελώς η έκφραση του γονιδίου αναφοράς (*gfp*) απουσία της τετρακυκλίνης θα πρέπει να καθοριστεί. Μια άλλη πτυχή που πρέπει να ληφθεί υπόψη είναι η πιθανότητα η τετρακυκλίνη να είναι τοξική για το *Plasmodium berghei* και αν αποδειχθεί κάτι τέτοιο επιπλέον έλεγχοι για το αν ανάλογα τετρακυκλίνης μπορούν να χρησιμοποιηθούν στη θέση της πρέπει να διεξαχθούν. Τελικώς θα πρέπει να διεξαχθούν πειράματα για να διαπιστωθεί εάν το σύστημα είναι λειτουργικό όταν χρησιμοποιείται in vivo.

Εάν αποδειχθεί ότι το σύστημα καταστολέα τετρακυκλίνης-Απταμερούς είναι απολύτως λειτουργικό για το *P.berghei* και ρυθμιζόμενο με τη συγκέντρωση της τετρακυκλίνης, η μεταφορά του στο *P.falciparum* μπορεί να δώσει ένα πολύ καλό εργαλείο προκειμένου να μελετηθεί η λειτουργία των απλοειδών γονιδίων που υπάρχουν σε αφθονία στο γονιδίωμα του είδους αυτού.

1. Abstract

Malaria is a mosquito borne infectious disease that can affect humans and other animals. This disease has always been severe for humanity since it is difficult to control its expansion and therefore malaria can easily become a widespread disease as history has shown so many times. The causative agent of malaria is a parasitic protozoan called *Plasmodium* that belongs to the phylum *Apicomplexa*, whereas its transmission vector is the female *Anopheles* mosquito. This invertebrate host transmits sporozoites, which is a motile infective form of *Plasmodium,* to the vertebrate host upon biting. Sporozoites travel to the liver through blood vessels and infect hepatocytes. There it reproduces asexually producing thousands of merozoites, which have a potential to infect red blood cells where the parasite initiates a series of asexual multiplication cycles resulting in new merozoites. Some merozoites have the potential to develop into immature gametocytes and subsequently to form gametes, after being uptaken by a new mosquito during a blood meal. Inside the mosquito's midgut the male and female gametes fuse and do so form an ookinete, which is a fertilized motile zygote. Ookinetes develop into new sporozoites which migrate to the salivary glands and so the cycle starts over again.

Plasmodium falciparum (*P. falciparum*) is the one of the four known human malaria parasites which is responsible for most virulent type of malaria worldwide. Therefore studying its genome could lead to novel approaches for developing vaccines to effectively treat malaria. Nevertheless, often researchers use *Plasmodium berghei*, a rodent malaria parasite, rather than *P. falciparum* since P. berghei is non infectious to humans whereas the genome organization and genetics between these two species is highly conserved.

Plasmodium parasites are haploid during all life stages, except zygote to oocyst, and therefore deletion of any essential genes would cause a lethal phenotype. For this reason the development of conditional knockdown-out systems in order to study the function of essential genes is required. This study aims in developing the Tetracycline Repressor-Aptamer system for *Plasmodium berghei.* The great advantage of this system is that protein levels can be regulated with tetracycline concentration. Aptamers are unique sequences that can bind small molecules or mRNA structures (Repressor) and thus interfere with gene expression. In absence of tetracycline the repressor can effectively bind to the aptamer and therefore gene expression is knocked down. When tetracycline is provided the repressor binds to the drug rather than to the aptamer, allowing normal gene expression.

For developing the Tetracycline Repressor-Aptamer system for *P. berghei* several transgenic control lines were successfully developed. First, two parasite lines expressing the Tetracycline Repressor under the control of either the HSP70 (constitutive expression of Tet Repressor) or the P25 (expression of repressor only post activation of gametocytes) promoter were generated and expression of the repressor was confirmed with Western blotting for both lines. Further on the line expressing the repressor constitutively was positively selected and cloned. This cloned line was then again tested with Western blotting to confirm the expression of the Repressor and was also tested for any growth defects and subsequently negatively selected in order to be used for further transfections. In order to test the effect of the Functional and Non-Functional Aptamer on the expression of the reporter gene (*gfp*) two parasite lines expressing either the Functional or the Non-Functional Aptamer in front of the *gfp* sequence were required. Only the *Non-Functional Aptamer-GFP* line was generated successfully, whereas for the *Functional Aptamer-GFP* line several attempts resulted in a transfection vector containing a mutated *Functional Aptamer*. Last,

one positive parasite line expressing only the *gfp* gene was successfully generated. All transgenic parasite lines were generated using a plasmid as a transfection vector and transfection occurred through a double cross over recombination event.

Future prospects for the Tetracycline Repressor-Aptamer system involve transfection of the *aptamer:gfp* vectors in the *Tetracycline Repressor* expressing lines so that four test parasite lines will be generated. After cloning these lines and testing them for any growth defects they will subsequently be used for testing the system in presence and absence of tetracycline. Also the reversibility of the system upon addition of tetracycline as well as the possibility to completely knock down the reporter gene (*gfp*) using this system need to be determined. Another aspect that has to be taken into consideration is whether tetracycline is toxic to *P.berghei* and if so further tests to determine if tetracycline analogues can be used instead need to be carried out. Last, experiments need to be done so as to check if the system is functional when used in vivo.

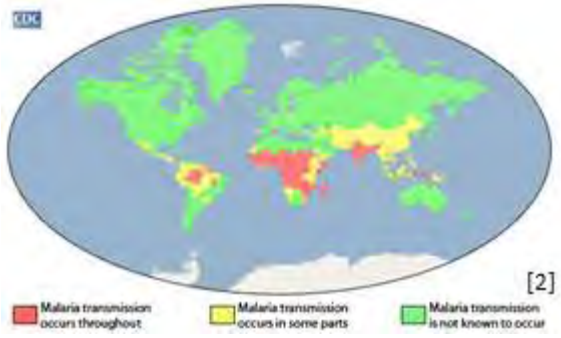
If the Tetracycline Repressor-Aptamer system proves to be totally functional and regulatable upon tetracycline concentration, adopting this system to *P. falciparum* will release another very effective conditional knockdown system for this species.

2. Introduction

Malaria: a widespread disease

Malaria is nowadays a widespread disease. There are several climatic factors that contribute to malaria being the queen of diseases in certain countries. Temperature, humidity and rainfall are some of these factors that help malaria to be transmitted mainly in tropical and subtropical regions. In these areas both Anopheles mosquito can survive and multiply and malaria parasites can complete their growth cycle in the mosquitoes. For that, temperature is the most important aspect because under 20 °C *Plasmodium falciparum,* which is the most common causative agent of malaria (see: Species of *Plasmodium* that cause malaria), cannot complete its growth cycle and thus cannot be transmitted. Generally, the highest transmission rates of malaria are found in Africa South of the Sahara and in some parts of Oceania including Papua New Guinea. In cooler territories transmission of malaria due to *P. falciparum* will be intense only during the hot periods, while *P. vivax* is more tolerant of lower temperatures and thus more prevalent there. In many developed areas as Western Europe or the United States economic development and public health measures have played a significant role in eliminating malaria incidents [1] .

Several factors can influence the transmission of malaria even inside the same malaria endemic country. For example in tropical and subtropical areas where malaria is widespread the disease does not occur at very high altimeter where the temperature is lower or during colder seasons. It does also not occur in deserts where climatic conditions are too severe for mosquitoes and Plasmodium to survive [1].



[2]

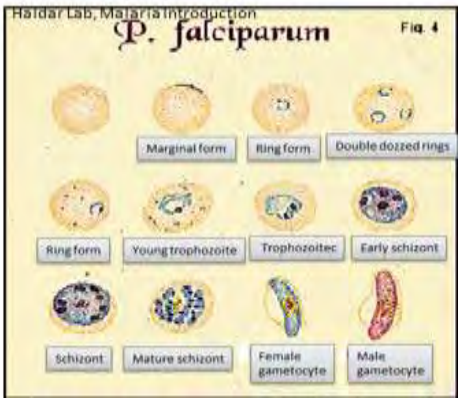
In the year 2012, 99 countries were recorded as countries with ongoing malaria transmission. Considering the fact that malaria was in a reintroduction phase in other 5 countries, there appears to be a total of 104 countries where malaria was considered to be endemic. When looking back at the year 2011, globally 3.3 billion people were infected with malaria [1]. It should be mentioned that approximately 80% of all malaria deaths are distributed in only 14 countries, most of which appear to be in Sub-Saharan Africa. It is also important to refer that 80% of malaria cases in 2010 were found in only 17 countries. Among the countries with the highest incident rate there are Congo, India and Nigeria [2].

Malaria is also widespread in other countries in Asia and Oceania including India, Pakistan, Bangladesh, Thailand, Vietnam, Laos, Myanmar, Cambodia, Indonesia and Papua New Guinea. This mosquito transmitted disease also seems to appear in certain countries in South America including Brazil, Peru, Colombia, Bolivia, Ecuador, Venezuela, Guyana, Suriname, and French Guiana. The most common species in these countries is *P. vivax*, but *P.*

falciparum seems to be on an increasing rate as well. There have also been recorded several cases of malaria in countries in Central America and Southern Mexico as well as in some countries of the Caribbean and North America. Europe seems to have a low rate of malaria incidents and only some countries are affected, including Turkey, Armenia and Azerbaijan[3] [4].

Species of *Plasmodium* that cause malaria

There are five species of *Plasmodium* that can infect and be transmitted by humans. The highest number of deaths is causes by *P. falciparum* and *P. vivax*. Particularly almost 80% of all malaria cases in Africa are caused by *P. falciparum*, whereas *P. vivax* is widespread in Asia and causes 95% of malaria cases there. Hypnozoites of *P. ovale* and *P. malariae* species can survive for years in the liver and cause a general milder form of malaria hardly ever fatal. There is also a zoonotic species of malaria that commonly infects macaques but can also infect humans. The most dangerous of these species appears to be *P. falciparum* which is most predominant in Sub-Saharan Africa. Therefore it has been recorded the 90% of the world's malaria cases occur in Africa [3].



Transmission and symptoms of malaria

Malaria is a mosquito borne infectious disease transmitted via the bite of a female Anopheles mosquito. Responsible for malaria disease is a protozoan parasite from the genus Plasmodium. When an infected mosquito bites an uninfected person parasites can travel from salivary glands of the mosquito to the liver of the bitten person. Inside the human body the parasite can multiple by infecting hepatocytes and after that infect Red Blood Cells (RBCs). The gravity of transmission depends on factors associated with the parasite, the host, the vector and the environment. Factors that affect the survival of mosquitoes play a significant role in transmission of malaria. Climatic conditions, such as rainfall and temperature can affect the life cycle of mosquitos and thus the mosquito stages of Plasmodium species. If such climatic conditions appear in areas where people have low immunity to malaria the disease is preferably transmitted [3].

Particularly the temperature of an area plays a significant role in malaria transmission. *Plasmodium* species which cause malaria cannot survive without the mosquito vector. Thus

mosquito population dynamics are an important factor for *Plasmodium* survival. Considering that the mosquito population and *Plasmodium* survival depends upon the temperature of a territory this environmental factor seems to appear very important for malaria transmission. Recently there were performed some experiments revealing that the juvenile mosquito stages are much more sensitive to temperature changes than previously thought. It is also believed that mosquitoes growing at certain age that is best for malaria transmission are more likely to be abundant at specific temperatures. These results indicate that it is of great importance to include vector biology when studying the reasons for malaria transmission extensively [9].

Symptoms of malaria include fever, headache, vomiting and chills which typically appear between 10 and 15 days after the mosquito bites a non- immune person. Children affected with malaria can often develop other symptoms as well, such as severe anaemia, respiratory distress or cerebral malaria. If left untreated the disease can easily become life threatening and can lead to coma or even to death. The reason for malaria being so severe is that once induced to human bodies the parasites can multiple and after a while disrupt the blood supply to vital organs. Asymptotic infections might also occur in regions where population has developed immunity against malaria [3].

Diagnosis of malaria

It is of high importance to diagnose malaria accurately and early enough so that the disease can be treated effectively. Misdiagnosis is very dangerous as it can lead to high morbidity and mortality. In areas where malaria is no longer endemic diagnosis of the disease might not be accurate because medical personnel does no longer consider malaria as a potential reason for illness and thus do not order necessary diagnostic tests. In some areas on the other hand, malaria cases are so intense that the largest amount of population is infected but remains asymptotic, e.g. Africa. These people have developed the needed immunity in order to be protected from the disease, but can still be infected. It is also very important to mention that in territories where malaria is endemic, usually there are also some other equally severe diseases, such as tuberculosis or HIV/AIDS. Thus health personnel cannot focus on malaria cases only and many of these cases remain untreated [10].

Usually the diagnosis of malaria is performed by a microscopic examination of blood [3]. Either blood films or antigen based rapid diagnostic tests can be used. Although it seems to be rather easy to detect parasite that way there is a number of factor that can make that procedure complicated. First of all there are 5 different forms of malaria species which need to be identified and distinguished one from another in a patient's blood in order to accurately diagnose malaria. Further on signs and symptoms need to be interpreted accurately so as the treatment will be specific. The percentage of parasitemia and of persisting viable or non-viable parasitemia is also an important factor in diagnosing malaria.

Last the sequestration of the parasites in the deeper tissues can lead to misinterpretation of the parasitemia and thus to the wrong kind of treatment [10].

The best technique and the most accurate one is the polymerase chain reaction technique which can detect the parasite's DNA. This technique has proved to be very efficient even if the parasitemia is very low. PCR can detect as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT. It can also be used in mixed infection cases. The disadvantage of this technique is that it is too expensive to be used in malaria endemic countries [11] [12].

Pregnancy can lead to increased danger of malaria infection due to failures of the maternal health. Thus pregnant women are a high risk group and should be examined for malaria frequently. The problem is that during pregnancy diagnosis of malaria can be difficult due to absence or low parasite density in peripheral blood and thus microscopic analyses of blood cannot be used[13].

High risk groups

Malaria can be more threatening to some specific population groups. Infants or young children under the age of 5 in areas where the population has not yet developed protective immunity are at a great risk of being infected. Adults over the age of 65 can also be included in the high risk groups since the immune system of these people might experience some default. Further on, non-immune pregnant women are also a target group for malaria transmission and in this case infection can also lead to miscarriage or even maternal death. When semi-immune pregnant women are infected the risk of losing a child is very high. Also people on long term steroids or people receiving chemotherapy can constitute a high risk group. When a person with HIV/AIDS is infected with malaria the danger of a potential death is even bigger since the immune system of this person has been weakened [3]. This also includes chronically ill people and people who had their spleen removed. Last, travelers also form a high risk group, since people travelling to malaria endemic countries are usually non immune and can be easily infected by the malaria parasite and manifest symptoms rapidly [14].

The categories mentioned above might be the high risk groups of people who can be infected with malaria, but that should not be misunderstood. Young, healthy people with a strong, well working immune system can be infected as well [14] .

Malaria in pregnancy

Every year approximately 30 million women in Africa and other malaria endemic countries become pregnant, raising the great issue of malaria prevention and treatment during pregnancy. Infection with *Plasmodium falciparum* , which is the most common causative agent of severe malaria, can have very sober consequences for mother and unborn child. The Roll Back Malaria partnership recommends three control methods in order to reduce malaria spread among pregnant women. First insecticide treated nets (ITNs) should be used among all pregnant women, especially in endemic countries. Second, individual and accurate treatment of each malaria detected case must be considered as a necessary procedure and should not be neglected. Third, in malaria endemic countries where stable transmission of malaria is detected, Intermittent Preventive Treatment (IPT) must be a priority.

In areas of low or epidemic unstable transmission the percentage of immune individuals is considerably lower than in endemic countries. In such regions non immune pregnant women are at a 2 to 3 fold higher risk of getting infected with malaria than other adults. In these cases the consequences for the pregnant woman can be cerebral malaria, pulmonary oedema, anaemia or hypoglycaemia. For the unborn child stillbirth or abortion can be the case. If the child survives low birth weight can be the case. In such areas of low transmission treatment of every acute incident of malaria could be the solution, since non immune pregnant women are on a high risk of developing clinical symptoms of malaria as mentioned above. Use of ITNs can also help to prevent spreading of the disease.

In areas of stable transmission the risk for a pregnant woman is not that high due to partial clinical immunity which these populations have achieved during many years of exposure to stable malaria transmission. The most common effects detected in malaria infected women in such regions are maternal anaemia and low birth weight. Nevertheless, 10,000 maternal deaths caused my malaria every year and 3-8% of infant mortality is noticed in such countries. In endemic areas with a stable transmission rate ITNs and IPT are most commonly used to protect pregnant women from malaria disease. Pregnant women might develop anaemia due to malaria even if no peripheral parasitemia is detected since parasites can sequester in the placenta. Therefore all pregnant women from areas with high transmission rates should be treated with an effective antimalarial drug.

It should also be mentioned that the risk for HIV infected pregnant women to develop clinical symptomatic malaria once infected with *Plasmodium falciparum* is even higher than for an otherwise healthy malaria infected pregnant women. This can be explained due to their weakened, impaired immunity system which does not allow them to fight malaria effectively [15].

Cerebral malaria

Cerebral malaria is the most severe neurological manifestation of malaria and causes brain injury to an unacceptable number of children. Coma or mortality is the most common outcome of that disease and even if the patient survives brain injury occurs. Cerebral malaria affects both children and adults but is most severe in young people. Its clinical symptoms include asexual forms of *Plasmodium falciparum* parasites in peripheral blood smears.

In the beginning it was difficult to discover the exact symptoms of cerebral malaria because patients experiencing other encephalopathies might incidentally also have parasitemia and thus were included to those suffering from cerebral malaria. This lack of specificity is problematic for clinical and pathogenesis studies. The most clear clinical symptoms for cerebral malaria is coma and impaired consciousness, which are thought to be caused by infected Red Blood Cells (RBCs) or altered metabolic pathways and factors. Other common symptoms are brain swelling, intracranial hypertension, retinal changes and brainstem signs. Further on, anemia and metabolic acidosis are commonly experienced in cerebral malaria patients as well as hyperpyrexia or hypoglycemia. As part of a multi organ disease, cerebral malaria patients develop fever, headache, body ache and in the end delirium and coma. Some infected people also develop hemoglobinuria, jaundice, shock, renal failure, lactic acidosis, abnormal bleeding, pulmonary edema and adult respiratory distress syndrome. Due to a default of the immune systems bacterial co-infections are also very common in cerebral malaria patients. The outcome of cerebral malaria is death in most cases, and even if a patient survives he sustains significant brain injury, especially children.

Sequestration of parasites in cerebral microvasculature is considered to be a central factor of cerebral malaria, but there are also a lot of other factors that are causative agents of this sever disease. RBCs have the ability to adherence to the endothelial cells using some proteins that emerge on their surface after contact with the parasite. These antigens adherence to intercellular molecular proteins which are upregulated in areas adjacent to sequestered parasites. Other RBCs can agglutinate with other RBCs and do so form sequestered parasites masses which impair perfusion and can so cause hypoxia and lead to coma.

Adherence of RBCs to cerebral endothelium cells of the host lead to a cascade of events. First, genes involved in inflammation are transcript, signal transduction and cell to cell signaling is mediated, release of endothelial microparticles (EMPs) is triggered and in the end apoptosis of host cells occurs. It has been noticed that in patients experiencing coma EMPs are increased. Further on, repair of injured endothelium cells is impaired since endothelial progenitor and plasma levels of endothelial regulators are not sufficiently provided. It has been showed in murine models that endothelium cells are first led to apoptosis and neurons and glia follow. The signal for apoptosis is the contact of RBCs with the endothelium. Perforin mRNA levels increase in cells that are led to apoptosis, and it has been noticed that in perforin deficient models cerebral symptoms and apoptosis do not occur. Thus perforin must play an important role in apoptosis.

It is normal that cerebral blood flow increases in patients with cerebral malaria because body tries to cover the metabolic demand and to provide enough oxygen and nutrient to tissues. In contrast to this it has been noticed that in patients suffering from cerebral malaria multiple areas of retina whitening occur due to under perfusion in those areas. When considering that the retina mirrors event in the brain, it is easily understood why small, multiple areas of under perfusion might occur in the brain as well. After that observation was made, it is believed that coma in cerebral malaria might be partially caused due to under perfusion of these areas. The reason why with early treatment of the disease tissue necrosis can be minimal is that the patches of brain are very small.

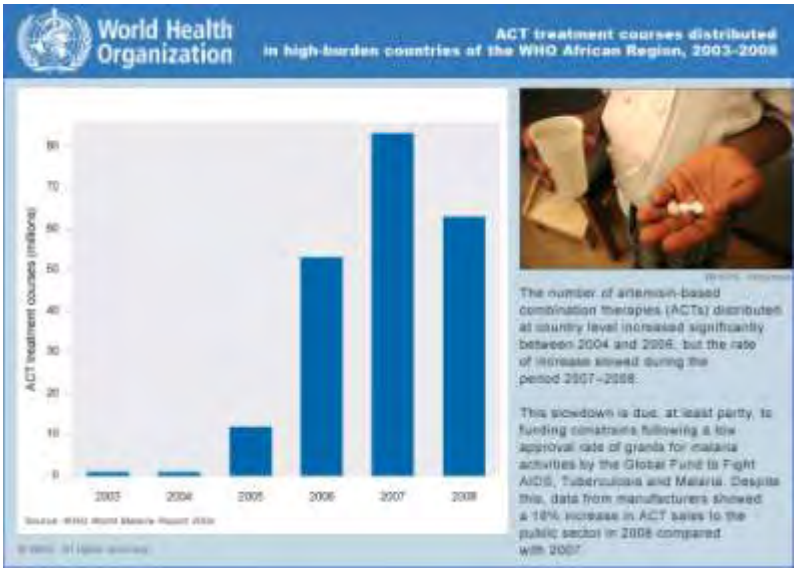
Another symptom of cerebral malaria is that there is an increased risk of epileptic crisis and seizures, which increased with parasitemia. Seizures are very common in childhood cerebral malaria and it has been noticed in many cases that prolonged seizures are followed by irreversible neuron damage. After some days edema is recognized and after that atrophy and gliosis are noticed. The question though whether seizures result from brain injuries or if seizures cause brain injury has not been answered yet. It is known that prolonged seizures might worsen a brain injury and so start a circle of more neural injuries and seizures.

No extensive studies focusing on cerebral malaria in children and sequelae of the disease have been carried out since mortality caused by malaria is a more intense problem. New methods of rehabilitation are needed as the number of children suffering from sequelae of cerebral malaria is on the increase. Rehabilitation should include physical and occupational therapy, behavior and speech therapy, cognitive rehabilitation and hearing aids [16].

Anti-malaria drugs

There is a great variety of anti-malarial drugs available nowadays. Which drug or drug combination should be used in each case depends on the physiopathology of the patient and the soberness of the incident. Uncomplicated malaria may be treated with oral medications. In cases of severe malaria it is very important that parenteral treatment is given along with oral treatment. There are two different groups of drugs available for parenteral treatment, the cinchona alkaloids (quinine and quinidine) and the artemisinin derivatives (artesunate, artemether, artemotil) [17].

Artemisinin and its derivatives are the most common used anti-malarial drugs nowadays. This group of drugs is extremely potent and has a rapid onset of action. When used together with other slower drugs very short courses of treatment can be highly effective. It is generally a very common



treatment method to combine anti-malarial drugs with completely different modes of action so that the causative parasite cannot develop resistance to different combination. Even in the case resistance against one anti-malarial drug is developed the other drug will still kill the parasite. Currently 5 artemisinin based combination therapies are recommended by the World Health Organization. What artemisinin derivatives do in such kind of treatments is to reduce the parasite load during the three first days of treatment, while the secondary drug eliminates all remaining parasites.[18] Artemisinin derivatives can also reduce the number of parasites by approximately 10,000 per asexual cycle and another very important feature of these drugs is that they have gametocidal properties. This means that they can reduce transmission by reducing the number of disseminated parasites or worse already resistant parasites. It has been proved that treatment with a combination of artemether/ lumefantrine is more rapid in gametocyte clearance than other antimalarial drug combinations. However recent reports from the Thai–Cambodian border reveal evidence of emerging resistance to artemisinins [18].

Artemisinin based combinations of anti-malarial drugs can also be used by pregnant women during the second and third trimester of pregnancy. Some of these combinations are artemether-lumefantrine, artesunate-amodiaquine, artesunate-meflowuine and artesunate-sulphadoxine-pyrimethamine. It should be mentioned though that those artemisinin derivatives are not recomented during the first trimester of pregnancy due to limited clinical safety data and evidence of embryo lethality and developmental abnormalities [19].

The use of quinine for treatment of severe malaria incidents was initiated long before modern clinical trial methods were developed. Some salts of quinine are still used for parenteral treatment with dihydrochloride being the most widely used. Quinidine on the other hand is much more toxic than quinine and is therefore used only if no other efficient parenteral drugs are available.

Non artemisinin based drug combinations include sulfadoxine-pyrimethamine (SP) plus chloroquine or amodiaquine. Parasites have developed a very high resistance level to those drugs and though have compromised their efficacy. The combination of chloroquine with SP is not more efficient that each drug alone, whereas the combination of amodiaquine with SP

might be more efficient that using every drug alone but is inferior to any artemisinin combination and thus no longer recommended for the treatment of malaria [17].

There is an emerging need for developing new anti-malarial drugs and since cases of relapsing malaria seem to become more and more frequent drugs to eradicate those cases are needed as well. Extensive research towards the direction of developing drugs that break down the lifecycle between human and mosquito host also needs to be done since this is the only way to bloke transmission of parasites. Recently four new fixed dose combination medicines have been approved. OZ439 is a synthetic endoperoxide that may be able to reduce treatment to only one dose. Spiroindolone, which is already in clinical development, kills blood stage parasites of both *P. falciparum* and *P. vivax* [20].

Huge efforts towards the development of transmission blocking vaccines have recently been made. For that specific molecules present only and highly conserved in the Anopheles species are used as a target for anti-malarial drugs. AnAPN1 is a promising transmission blocking vaccine that has the ability to target a surface antigen on the midgut of Anopheles mosquito. It has been revealed that antibodies targeting this drug can block transmission by only single epitope recognition. Thus AnAPN1 seems to be a very promising vaccine for malaria. Other transmission blocking vaccines are being developed as well and appear to be promising [21].

Resistance to anti-malarial drugs

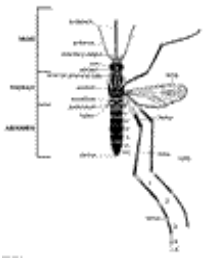
There has been a great issue rising about resistance to antimalarial medicines. Previous generations of medicine, such as chloroquine and sulfadoxine-pyrimethamine are no longer efficient as *P. falciparum* was able to develop resistance in the 1970s and 1980s. *Plasmodium falciparum* also seems to develop resistance to the nowadays used antimalarial drug artemisinin. So far 4 countries in South East Asia have been detected that show to have developed resistance to artemisinin. The reason for this might be that patient taking oral treatment of artemisinin may discontinue treatment premature as the symptoms of malaria might disappear rapidly. This means that the parasites in blood might survive and can so be passed to another mosquito. Here is a map of countries that have already developed resistance to one or more antimalarial drugs [3].

The consequences of developing resistance to artemisinin derivatives are very severe and should not be ignored. Patients that are treated with an artemisinin based combination therapy still recover and so resistant parasites are no prevented from spreading. Even more severe is the possibility for these parasites to develop resistance to all available anti-malarial drugs and so limit to combination of drug treatment that could be used. In western Cambodia combinations of artemisinin with other effective anti-malarial drugs has become so rare that a non artemisinin based combination therapy, atovaquone- proguanil, is used currently [18].

The World Health Organization needs to take into consideration the possibility of an already developed resistance to the partner drug used along with an artemisinin derivative. If parasites have developed resistance to one drug it is even more likely to develop resistance to the partner drug, so artemisinin derivatives should be used only together with a partner drug to which parasites do certainly show no resistance. If failure rates of treatment are higher than 10% then the WHO recommends the endemic countries to switch to another partner drug [18].

Malaria Vectors

Malaria is transmitted among humans by female mosquitoes that belong to the genus *Anopheles. Plasmodium* change host, from the vertebrate to the invertebrate and vice versa when a female *Anopheles* mosquito takes a blood meal. Inside the invertebrate host gametocytes of *Plasmodium* get activated and change into gametes. After fusion of male and female gamete the zygote which is formed undergoes several changes and results in becoming sporozoites, which is the motile invasive form the parasite uses in order to migrate to hepatocytes of the vertebrate host.



Approximately 3,500 species of mosquitoes exist and are grouped into 41 genera. As mentioned above human malaria is transmitted exclusively by the female *Anopheles* mosquito, but is has to be mentioned that from the 430 *Anopheles* species only 30-40 transmit malaria (i.e. are “vectors”) in nature. The female *Anopheles* mosquito can be found worldwide except Antarctica. In different regions and environments different *Anopheles* species transmit malaria, depending on the climatic conditions. Finally it should be mentioned that Anophelines that transmit malaria are not only found in malaria endemic countries but also in areas where elimination of malaria has proved successful. Therefore all these areas are at a constant risk of re-introducing malaria.

Vector control methods

In order to fight malaria worldwide effective methods for controlling the vector have to be used. Vector control methods have been proved to significantly reduce the interrupting disease transmission when coverage is high enough. The two most common vector control methods are indoor residual spraying (IRS) and long- lasting insecticidal nets (LLINs). It is also important to make the indoor places as much inhospitable to mosquitoes as possible [3].

IRS is the adjustment of some chemicals that are usually long lasting on every surface of a house and domestic animal shelters. Any mosquito vector that lands on that surfaced will be

affected or killed by the exposure to the chemical. In this way both the life span and the density of the vector is reduced efficiently. There have also been noticed some cases in which the mosquito vector was even completely eliminated by IRS. Indoor residual spraying can effectively reduce and interrupt malaria transmission. It was not used extensively in recent years and was brought back to use in countries where it was implemented well. IRS should be combined with residual pesticides such as DDT and this combination should play a central role in down regulating malaria worldwide. There are however some issues about this vector control method that should be taken in consideration. First of all unauthorized or recommended pesticides should be strictly prevented and it is also vital to impede insecticide resistance. Research towards the direction of new insecticides that are more efficient and long lasting need to be done [22].

The other strategy of vector control is using insecticide treated nets. These nets have been used for many years, since the Second World War when Russian, German and US armies treated these nets with residual pesticides. In the late 1970s synthetic pyrethroids were used for the first time in combination with bed nets. The great advantage of pyrethroids is that they have a very low mammalian toxicity and can so be used without fear. The great question about mosquito bed nets was whether they can reduce the number of children dying from malaria. One report carried out in Gambia showed that there was a 42% reduction in mortality in children from 1 to 59 months when bed nets were used systematically. There appears to be increasing funding towards the direction of vector control methods. The proportion of households owing at least one insecticide treated mosquito net (ITN) shows to increase dramatically from 10% in 2005 to 53% in 2011. There was also an increase in the number of people sleeping under a mosquito net at the same time period [2].

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Economic impacts of malaria

During the latest years it has been noticed that the gap in welfare between countries experiencing malaria and those not affected with malaria is expanding. Historically, malaria has been responsible for a growth penalty of up to 1.3% per year in some African countries. It has also been noticed that annual economic growth is on the border of been very low in countries where malaria transmission is high. The World Health Organization reports that most of the deaths caused by malaria occur in poor countries of the tropics and Sub-Saharan Africa. It has also been proposed by several researchers that ecological impacts that contributed to the expansion of malaria are perhaps some of the most important reasons for some countries to experience poverty nowadays. The question that has been posed many times is if malaria leads to poverty or if poverty is a causative agent for malaria. It seems that this correlation goes in both directions [6].

Malaria causes morbidity and mortality and thus has direct economic consequences. The direct cost of the disease includes both individual and social expenditures. The economic impacts on individuals include the value of lives lost, the value of time lost due to sickness, and the expenditures on medical care, treatment and prevention. Malaria affected people also need to be transported to the nearest medical facility and sometimes a member of the family needs to stay with the hospitalized person. Also societies and governments spend large amounts of money on malaria control and prevention. Governments need to maintain health facilities and health care infrastructures, education and it is also of great importance to sustain research programs. All these are a great challenge for the Sub-African countries and most other countries affected with malaria since public recourses are very limited [6][7].

Indirect cost includes changes in human settlements as well as work patterns induced by the disease. This means lost productivity or income due to lost workdays or days of absenteeism from work. Also the work done at home by all family members is affected by the illness. If death occurs the lost includes the future lifetime earnings of those people and not to mention human suffering when losing a family member. The disease has also consequences on fertility, demography and capital investments. Therefore malaria is associated with many variables that are correlated with income levels [6, 7].

Another problem malaria endemic countries experience is that the disease influences social and economic decisions. Investors are often prevented from the risk of contracting malaria in such countries and do so prefer other countries for their investments. The same thing happens with researchers, teachers, doctors and other specialized stuff resulting in underdevelopment of education, health patterns and research programs. Tourism also suffers in countries where malaria is widespread since tourists are threatened by a potential malaria infection.

In very poor territories education suffers most times. Families need their children for work and therefore the percentage of children visiting school permanently is extremely low. Another reason why people in poor region remain uneducated is that education might not reach these places. Education levels are highly correlated with malaria because illiterate people or people with low levels of education might not be able to understand written health education materials, like posters or flyers. Further on, information does sometimes not reach poor territories, for example poor households might not have access to television or radio and are therefore missing health messages broadcast through these media. It should also be mentioned that women, who are those responsible for rising up children and who take care of them during the first years of life are even more uneducated and therefore uninformed about malaria disease. Thus, although health information about malaria patterns is available even in very poor countries they are not really made available for uneducated people and so societies cannot benefit from that information. It is so made clear that low levels of education are equal to low knowledge of malaria in most cases[8].

It seems that all co-causes that help malaria to be a worldwide disease are related with poverty and thus the most effective way of fighting the disease is to fight poverty and to increase economic levels in many African and Asia countries[8].

History of malaria

The earliest reports of malaria cases are found at around 4,000 years ago. Malaria originates from the Italian words ''bad air'' because it usually appeared at places with swamps. When considering that ancient human societies had given a name to that particular disease and that this disease was mentioned in many manuscripts, someone can conclude that malaria must have influenced human populations and human society to a great extent.

Malaria was first mentioned in Chinese medical writings. The Emperor Huang Ti edited the medical book Nei Ching, where in 2700BC several symptoms of malaria were mentioned. Malaria was also very common in Greece during the 4th century BCE. Hippocrates first noted the most common symptoms of malaria and by the age of Pericles, malaria was mentioned in many manuscripts. In Greece malaria also played a significant role in the decline of rural areas where the disease was more frequent. It was not until the finding of a Sanskrit medical treatise when malaria was attributed to the bites of certain insects.

The Qinghao plant was first mentioned in a Chinese medical treatise and in 340CE the antifever properties of that plant were revealed. The ingredient of Qinghao that can actually down regulate fever is Artemisinins and it was isolated by a Chinese scientist in 1971. Artemisinins and derivatives are even used nowadays very extensively and are considered as very effective antimalarial drugs.

The next anti-malarial drug used was Quinine. When Spanish Jesuit missionaries arrived in the New World they learned how to use a medicinal bark for fever treatment. This bark derived anti-malarial medicine quickly became very popular and even the Countess of Chinchon was cured of her fever. The bark from that tree, which was name Chincona after the Countess, in nowadays a very effective and popular anti-malarial drug used worldwide.

The first parasites in blood of a malaria patient were noticed on the 6th of November 1880 from a French army surgeon stationed in Constantine, Algeria. His name was Charles Louis Alphonse Lavern and he was awarded with the Nobel Prize in 1907.

Camillo Golgi was the first to propose that there must be at least two different forms of malaria disease, one with quartan periodicity and one with tertian periodicity. Another very important finding made by this Italian neurophysiologist was that fever does not occur until rupture and release of the parasites into the blood stream. For these discoveries he was awarded the Nobel Prize in Medicine in 1906.

The malaria parasite's first names were *Plasmodium vivax* and *Plasmodium malariae* which were given to them by two Italian investigators Giovanni Batista Grassi and Raimondo Filetti. It was not until 1897 when *Plasmodium falciparum* got its actual name by William H. Welch.

The Italian investigators Giovanni Batista Grassi and Raimondo Filetti first introduced the names *Plasmodium vivax* and *P. malariae* for two of the malaria parasites that affect

humans in 1890. Laveran had believed that there was only one species, *Oscillaria malariae*. An American, William H. Welch, reviewed the subject and, in 1897, he named the malignant tertian malaria parasite *P. falciparum*. This name was not at once accepted as there were many arguments about it, but the use of it in literature became so extensive that a change back to the name given by Laveran was no longer possible. It was in the year 1922 when the four human malaria parasites were described, *P. vivax*, *P. falciparum*, *P. ovale* and *P. knowlesi*. *P. knowlesi* was first found in a long tailed macaque, while the first incident of human infection by this parasite was described in 1965.

Until then the problem of malaria transmission still remained unsolved. On August 20th 1897 a British officer in the Indian Medical Service, Ronald Ross, discovered that malaria disease could be passed from malaria patients to mosquitoes during blood meal and then again to people by a mosquito bite. For doing so parasites needed to stay inside the mosquitoes for some time, which was called the sporogonic cycle. The whole sporogonic cycle of *P. falciparum*, *P. vivax* and *P. malariae* was revealed at around 1899, when a team of Italian investigators let some Anopheles mosquitoes to feed on malaria parasites.

The construction of the Panama Canal was at first prevented by yellow fever and malaria incidents which were very abundant among workers. By 1912 the number of hospitalized workers declined rapidly due to control programs for insects and malaria.

Another effective anti-malarial drug that was discovered in 1934 by a German is chloroquine, which was at first named resochin. It was not until 1946 that the drug was established as effective and safe by British and US scientists.

Dichloro-diphenyl-trichloroethane, which is a highly toxic substance, was synthesized by a German Chemistry student in 1874. It was extensively used as an anti-louse drug during WWII by various militaries. Later on it was also used as an antimalarial drug. Nowadays its use as a human used drug is no longer allowed as it was proved as highly toxic and carcinogen [5].

In 1955 at the World Health Assembly the World Health Organization presented a very ambitious suggestion for eradication of malaria worldwide. The first eradications efforts were carried out then with house spraying with residual insecticides, with antimalarial drug treatment and surveillance. Maintenance would also be at great importance for a successful eradication of malaria. The disease was successfully eliminated in many countries with mild climates and seasonal malaria transmission, whereas in other countries such as India and Sri Lanka the cases of malaria were down regulated at first but after efforts ceased they increased again. It should also be mentioned that most of the sub- Saharan countries were excluded from the eradication efforts.

At that present time many hindrances make it very difficult for the eradication campaign to be successful. The emergence of drug resistance, widespread available resistance to available insecticides, wars and massive population movements were some reasons why the campaign did not obtain long lasting funding from donor countries, so in the end efforts for complete eradication of malaria were given up. The new goal is now to introduce malaria control methods so that the disease will not spread un- inspected [11].

Latest news are encouraging

Fortunately, there is also some encouraging news. When taking a look back at the last decade malaria incidence and mortality cases have been reduced all over the world. In 2010 there were 216 million cases of malaria recorded and 655.000 malaria deaths which is 36.000 lower than the previous year. So there appears to be a 5% decline from year to year [3]. Of the 58 countries where malaria is systematically inspected, 50 are very likely to achieve the 75% reduction in malaria cases in 2015, compared to 2010. Four countries have recently completely eliminated malaria, the United Arab Emirates, Morocco, Turkmenistan and Armenia. Also ten countries are currently in the malaria elimination phase and another eleven in the pre- elimination phase[2]. Despite these encouraging facts the mortality rates caused my malaria are still far too high for a disease that is totally preventable and treatable, and as Raymond G. Chambers, the UN Secretary General's Special Envoy for malaria says '' one child still dies every minute from malaria and that is one child and one minute too many'' [3].

Plasmodium berghei's life cycle [23]

Sporozoites invasion and pre erythrocytic development

An infection starts when sporozoites present in the salivary glands of the invertebrate host, which is the female Anopheles mosquito, are released into the blood stream of the vertebrate host, the rodent. Once present in the blood of the rodent sporozoites can travel to the hepatocytes and invade them. For doing so two of the key proteins conserved between human and rodent malaria parasites play a significant role, the CS and the TRAP surface proteins. The host cell membrane forms a parasitophorous vacuole that allows the sporozoites to migrate through several cells before resting in one hepatocyte. Inside this final invaded hepatocyte sporozoites undergo the trophozoite stage and the schizont stage within 47 to 52 h. Nuclear division starts 24h after hepatocyte invasion and so the mature pre erythrocytic schizont can give rise to 1500-8000 merozoites. After rupture of the pre erythrocytic schizont, merozoites are released in the blood stream and can now infect RBC (red blood cells) [23].

Hypnozoite stage

This stage involves the arresting of sporozoites that have invaded hepatocytes in those cells for a prolonged period of time. Hypnozoite stage does not appear in *Plasmodium berghei*, but has been observed in some human malaria parasites such as *Plasmodium vivax* . Despite this stage not appearing in rodent malaria parasite it has been observed that *Plasmodium berghei* sometimes undergoes a long liver schizont stage due to slow development of liver schizonts.

Asexual development of erythrocytic parasite forms

Once released into the blood, merozoites can invade RBC. It has been observed that *Plasmodium berghei* merozoites have a preference for reticulocytes (young RBC), but can also invade mature RBC. Inside the RBCs merozoites undergo the ring stage and do so reach the trophozoite stage, which is characterized by an increase of the parasites size. Trophozoites are also characterized by a lot of granules inside the cytoplasm which are crystals of haemozoin. These crystals originate from the consumed haemoglobin which the trophozoites use in order to grow in size. 16h after invasion of RBCs trophozoites start duplicating their DNA and do so enter the schizont (Greek: tear apart) stage. The schizont stage lasts 6-8h and during that stage the nucleus of the parasite is divided lots of times and the DNA is duplicated several times. It is not until the end of schizogony that the parasite starts divided its cytoplasm and to form individual uninuclear merozoites. It is also worth to mention that different stages of blood parasites do simultaneously appear in blood and thus why erythrocytic development of *Plasmodium berghei* is considered as asynchronous. Once

the erythrocytic merozoites are released in blood stream they can infect new RBC and the cycle starts over again.

Before rupture of schizonts it has been observed that both mature and immature erythrocytic schizonts disappear from the peripheral blood stream and sequester in the capillaries of several inner organs. Parasites have therefore been detected in the liver and the spleen of the vertebrate host. Some surface proteins and some antigens of the RBC have been detected and it is believed that they might play a role in sequestration of the schizont to inner organs.

Sexual development

There is evidence that a percentage of 5-25% of each asexual cycle *Plasmodium berghei* fulfills inside the blood changes course and enters the sexual cycle. It is not exactly known when commitment of erythrocytic merozoites of *Plasmodium berghei* species to become gametocytes, which are the precursors of the mature gametes, appears. It is though believed that commitment might appear 12-16h after invasion of a RBC, at the trophozoite stage. It is most likely that at that time point trophozoites are committed to become gametocytes. Each erythrocytic schizont has commitment to give either only sexual or only asexual parasites and each individual sexually committed schizont gives progeny of only one sex, either male or female gametocytes. It should here be mentioned that in *Plasmodium falciparum* liver merozoites are already committed to become gametocytes at that early stage. Another difference of *Plasmodium falciparum* sexual development compared to that of *Plasmodium berghei* is that in *falciparum* pure cycles of asexual development appear and at some time point only sexual parasites are developed.

At the first 12-18h of sexual development, the trophozoites committed to become gametocytes have exactly the same developmental appearance as the non-committed trophozoites have. It is only after 16-22h that some sex specific features appear, but still male and female gametocytes cannot be differentiated. After 24h the male gametocytes start to change and to develop some male specific features. This involves less osmiophilic bodies, less dense stained cytoplasm due to less granules. The whole sexual cycle in the vertebrate host last up to 26-30h, which is very short when taking into consideration the time *Plasmodium falciparum* requires for sexual differentiation, 8 to 11 days!

The factors and the mechanism that are responsible for switching from asexual to sexual cycle in *Plasmodium berghei* are unknown so far. It is believed that environmental factors can play a significant role in asexual differentiation. Also there is some evidence that environmental factors in blood can influence the percentage of male to female gametocytes in blood. Another observation to mention is that unlike *Plasmodium falciparum* gametocytes that sequester in blood capillaries, there is no specific evidence that *Plasmodium berghei* gametocytes also have the same practice.

Another observation made for malaria parasites is that they lack sex chromosomes and so one asexual parasite can transform both in male and in female gametocyte. Thus malaria

parasites are considered as hermaphrodites that can in the absence of other parasites self-fertilize.

Gametogenesis, zygote development and ookinete formation in the mosquito's midgut

During a blood meal several forms of parasites are up taken from the female Anopheles mosquito and do so enter the mosquito's midgut. It should though be mentioned that only mature gametocytes can undergo further development in that specific environment. There have been noticed 3 environmental factors which trigger gametogenesis. These involve a drop of temperature to at least 5 °C, a rise of the PH from 7.3 to 7.8-8.0 and the presence of xanthurenic acid that lead to gametogenesis through calcium and cGMP-dependent signaling pathways. For the male gametocyte formation of gametes is a rapid procedure that takes approximately 10m. During this period the DNA of the male gametocyte is tripled and 3 round of nucleus division occur so that after that 8 haploid male gametes are formed. The male gamete undergoes a very interesting procedure which is known as exflaggelation. During exflaggelation 3 rapid rounds of endomitotic replication occur and 8 haploid male gametes are struggling to come out of the RBC. These gametes, which appear to be extremely motile, can stick to uninfected erythrocytes so that gametes can feed on them and so continue movement. The female gametocyte on the contrary forms into one single rounded haploid female gamete, the macrogamete. The large difference detected in appearance of male and female gametes can be explained due to the different developmental role they play in parasites life cycle. The male gamete is an ephemeral structure that has to be motile whereas the female gamete is a rather permanent structure suitable for establishing infection is the mosquito's midgut.

Fertilization occurs when the male gamete penetrated the cell membrane of the macrogamete and their nuclei fuse. The now formed diploid zygote undergoes meiosis so that the nucleus of the zygote contains now 4 times the haploid amount of DNA. The spherical zygote starts developing into the banana-shaped ookinete (Gr: moving egg) within 18-24h. The ookinete has a structure that resembles the structure of the other zoites, merozoites and sporozoites. This can be explained due to all zoites being invasive forms, so the apical complex that can be noticed in all of these forms helps them penetrate and traverse host cells. Further on the ookinete's pellicle is surrounded by another membrane that protect the ookinete from the mosquito's midgut where digestive proteases resident.

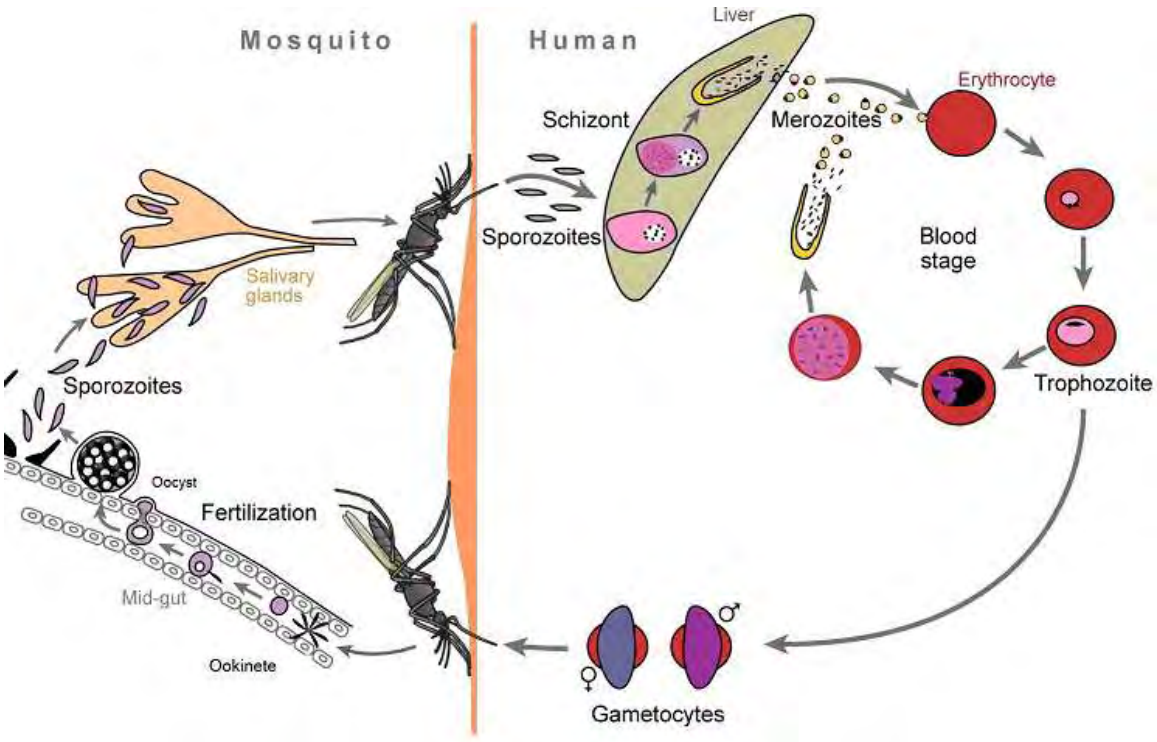
It is remarkable that although Plasmodium species are evolutionary altered, the transformation procedure inside their invertebrate host is highly conserved, showing the importance of this step in parasite life cycle.

Oocyst formation and the emergence of sporozoites

The banana-shaped ookinete is an invasive form as mentioned above and can though successfully invade epithelium midgut cells and make attachment with the basal lamina. The ookinete has not the ability to penetrate the basal lamina so it settles there attached to the basal lamina. There is no evidence that the ookinete does show preference for a specific cell type. The ookinete does traverse several cells before attaching to the basal lamina and it has been proven that those traversed cells are lead to apoptosis.

Once the ookinete has reached the basal lamina it starts reshaping to become a mature round Oocyst. This Oocyst initially grows asexually within 11-13 days and after that phase several mitotic replications occur that result in an increase of the oocyst's size and maturation. This mature Oocyst does now contain approximately 8000 haploid daughter cells, the sporozoites. Sporozoites are also invasive forms and do so have the characteristic apical complex shape all zoites (merozoites, sporozoites and ookinetes) possess.

After rupture of the mature Oocyst sporozoites are realized into the haemocoel and can invade mosquito's salivary glands. It has been shown that only 2% of the oocyst's sporozoites can reach salivary glands. Sporozoites do not stay inside the salivary gland cells, but they migrate through them and rest in the extracellular secretory space. For the parasites transmission it is very important that sporozoites can survive in the extracellular secretory space for several weeks before invading a new vertebrate host. Once the female Anopheles mosquito finds a victim sporozoites are injected in the vertebrate host. It has been shown that only 20-50 sporozoites per bite can be transmitted to the vertebrate host.



Why to use *Plasmodium berghei* for lab approaches

Although that the causative agent of most human malaria incident appears to be *Plasmodium falciparum* it is *Plasmodium berghei* that is used for most lab approaches, which is a rodent malaria parasite. There are several reasons why to use *Plasmodium berghei* rather than *Plasmodium falciparum* in lab approaches.

One of the most important reasons is that *Plasmodium berghei* is non- infectious to humans and does so not raise any safety patterns when it is used in labs. Further on there are several techniques for genetic modification available for berghei which means that the function of a lot of genes can be studied extensively. Also the three participants of rodent malaria, *Plasmodium berghei*, the rodent and Anopheles mosquito can all be genetically modified and their genome is known.

It should also be mentioned that extensive studies on *Plasmodium berghei* are preferable that on other Plasmodium species since the basic biology of human and rodent malaria is similar and molecular basis of drug resistance and sensitivity is also alike in human and rodent malaria. This means that if a drug against rodent malaria is developed this same drug could also be the basis for a drug against human malaria. Also the genome organization and the genetics are highly conserved between this two species.

Other reasons for using *Plasmodium berghei* in the lab are that the in vitro cultivation of sexual stages is easy and that *Plasmodium berghei* can be employed for the study of in vivo host parasite infection.

Development of conditional knockdown-out techniques for *Plasmodium* is essential

Recently researches are trying a new strategic in order to study *Plasmodium* so as to come up with novel ideas for developing malaria vaccines. This strategic involves the study of *Plasmodium* genes that are essential for its sexual stages development inside the invertebrate host so that genetic modification of *Plasmodium* including disruption of these genes could possibly block transmission. The problem the researchers are confronting is that disruption of many of these essential genes leads to a lethal phenotype at an early developmental stage and so the possible dual role of these genes latter on cannot be studied.. This happens because *Plasmodium* is haploid during almost all life stages and therefore deletion of any of these vital genes leads to death.

In order to circumvent this problem researchers try to develop conditional knockdown-out systems for *Plasmodium* species so that expression of any gene can be switched on and off at any time point.

Conditional knockdown- out- side techniques available for *P. berghei*

Swap promoter [24-26]

This technique involves the exchanging of a gene specific promoter with another one through homologous recombination. It is used in cases where a stage specific gene needs to be expressed at another developmental stage at which it is normally not expressed. This technique makes it possible for researchers to study the role of a gene at any developmental stage. It also makes it possible to study the function of a gene at a later developmental stage even if it might be essential at a previous stage. For this the gene of interest needs to be expressed under the control of a promoter that is functional only at the earlier developmental stage at which the gene product is needed, whereas at the later stage at which the gene function is unknown the promoter is not functional.

For swap promoter technique a vector is required. This vector needs to carry the required promoter between two regions which will be homologous to the 5' UTR regions flanking the endogenous promoter which needs to be exchanged. Three pairs of promoters are needed for this, two to amplify the two homologous regions and one to amplify the promoter that will be exchanged with the endogenous one. The vector also needs to carry a selective marker so that it will be easy to detect in which cell lines the exchanging of the promoter was successful.

Although swap promoter seems to be a good technique for conditional knockdown and has shown to work well in *P. berghei* it has some non-negligible drawbacks. Leakiness might appear due to protein carried over from earlier developmental stages at which the promoter is functional. In that case the gene might not be expressed at the specific stage that has been chosen but the phenotype will not be representative since protein will be still there and functional. Another drawback of that system is that different levels of leakiness might be experienced depending upon the specific promoter that is used in each case.

Protein destabilization domain [27, 28]

This conditional knockdown system has shown to work well in *Toxoplasma gondii*. It has also been applied to *Plasmodium falciparum* where it seems to work well but it still needs to be applied to *Plasmodium berghei*.
It is a reversible and rabid conditional knockdown system which involves the fusion of an $FKBP_{12}$ degradation domain to the protein of study. Upon fusion with this degradation domain the protein is led to proteasomes and is post translational degraded. It has been shown that degradation of a protein when using the FKBP domain is very quick, but time scale is affected by a lot of factors. The time required for degradation is very different depending on if the FKBP domain is fusion to the C terminus or the N terminus end of the protein. Also the time is affected by the specific protein which is getting degraded. A great advantage of this system is that it is reversible. A rapamycin analogue called Shld1 has the ability to bind to the FKBP domain and to protect the protein-FKBP complex from degradation. This reversibility of this system is also very quick as it has been shown in resent

studies. The levels of protein that are expressed normally and not getting degraded can be regulated upon the concentration of Shild1.
It has been shown that this system can be used in order to degrade native proteins in *Plasmodium falciparum* and thus is considered as a powerful tool for conditional knockdown in this species. It is also believed that it could be possible to integrate the FKBP domain at the 3' end of the endogenous coding region and to generate a conditional knockdown system is situ. This system seems to work better that the Tet- regulatable system since it does not depend upon the right timing or the specific promoter which has been used for tetracycline expression.
One of the limitations of the system is that leakiness might appear since the amount of the protein which is getting degraded is not always enough to produce the knockdown phenotype. Another limitation is that some proteins do not function well or do not function at all when made as fusion proteins. Mistargetting of the protein might also appear and pose a drawback of this system. It also should be taken into consideration that this system does only work well if the protein of study is cytoplasmic since the proteasomes are present only there. Last, some proteins are not getting degraded well upon fusion with the FKBP domain. For these proteins the system should not be applied.

RNA interference (RNAi) [29]

RNA interference is a very common technique of conditional knockdown. It is quick and reversible and therefore preferably used in order to study the function of genes by down regulation. It has been proved that this technique works very well in *Toxoplasma gondii* but all efforts to make it applicable for Plasmodium species have been unsuccessful until now.
This conditional knockdown technique is an endogenous mechanism of many organisms with which they regulate endogenous gene expression, silencing of transposable elements and repetitive genes or it can also be used as a defend against viral attack. Researches use the machinery of these organisms in order to specifically down regulate genes under study. For that, dsRNA having the sequence of the gene that needs to be knocked down is directly inserted though electroporation into the cells. Once present in a cell the dsRNAs are cut by the endogenous highly conserved enzyme Dicer to produce 21-25 nucleotide long RNAs. These RNAs are then unwilled by another endogenous enzyme complex called RISC. After becoming monoclonal, the RNAs specifically target endogenous genes being leaded by the enzyme Argonaut. The whole enzymology of this endogenous mechanism is highly conserved. Another way to down regulate specific genes with RNAi is to directly add siRNA into the culture medium.
Although this technique appears to be promising there is doubtless evidence that it does not work in Plasmodium species. All attempts to down regulate any blood stage gene, essential or not using RNAi in Plasmodium species were unsuccessful. It has been shown by phylogenetic analysis that Plasmodium species do not contain any of the highly conserved domains of the enzymes that complete gene silencing through RNAi. Even when the E- value was set very high Dicer and Argonaut highly conserved domains were not found in Plasmodium species. This indicated that Plasmodium lacks the required enzymology to complete RNAi. Further on no miRNAs were found in these species, and since miRNAs are an evidence of the endogenous RNAi mechanism this poses another evidence that Plasmodium species do not sustain an RNAi mechanism. On theory is that Plasmodium genome does not possess retrotransposons or viral pathogens, and thus there might not be selective advantage to the malaria parasites in either retaining a RNAi machinery or not.

Although all evidence show that RNAi does not occur in any Plasmodium species the abundant native antisense RNAs found in the cells leave open the question whether there finally might be a specific gene silencing mechanism. For this reason, and despite the evidence showing the opposite, further investigation of whether this endogenous mechanism appears in Plasmodium species or not needs to be done.

Transcriptional knockdown by Antisense RNA [30]

This conditional knockdown system is a very powerful tool of Molecular biology for studying gene function very specifically. It has shown to work well in many organisms, but there is still doubt about its efficacy and specificity in Plasmodium species.

It involves the introduction of a vector into the cell line of study. This vector conducts a selective marker, usually the DHFR gene, and a gene encoding the antisense sequence of the endogenous gene under study. Under the selective pressure of pyrimethamine only cells which have up taken the vector will survive. In these cells the antisense RNA will be expressed and will block the translation of the endogenous mRNA. Blocking of translation can occur due to hybridization between the endogenous mRNA and the antisense RNA, inactivation of the endogenous mRNA by ribonucleases activated by double stranded RNA or post transcriptional inhibition. The antisense RNA can also facilitate unusual splicing of the mRNA or inhibit normal transport of the mRNA.

The great advantage of this conditional knockdown system is that it is reversible. When drug pressure is removed loss of the episomally carried vector is facilitated and the endogenous gene that was silenced can be expressed again. This is a very good way to check if the phenotype caused by the antisense RNA of the vector is due to silencing of the specific gene of study rather than silencing of any other gene. In order to make this system even more time specific the antisense RNA gene could be posed under the control of a tetracycline regulated promoter. Another very great advantage of this system is that the desired phenotype can first be determined 6 weeks after the vector is introduced into the cell lines. As current targeted gene disruptions (TGD) in *Plasmodium falciparum* require 4 to 8 months of continuous culture, this alternative method that may more easily and rapidly lead to a phenotype is of very great interest.

Cre recombinase/ loxP system [31, 32]

Site specific recombination techniques, such as Cre recombinase/ loxP system, have become very important tools for conditionally and in vivo manipulating eukaryote genomes throughout the last years. Although this system seems to work well in most eukaryotes it has not yet been applied to Plasmodium species. There are made current attempts to introduce this system to Plasmodium species as well. Once developed this system would allow the study of the role of haploid genes through conditional knockdown in Plasmodium species.

For this system the 38kDa Cre recombinase of bacteriophage P1 is used. This enzyme can catalyze the recombination between two loxP sites, which are 34 base pairs specific recombination sites. The recombination can either be intermolecular or intramolecular. When an intermolecular recombination occurs a specific region of DNA located between two loxP sites can be inserted into the genome at a loxP site. Due to this intermolecular insertion mechanism being so specific the position effect is limited to the minimum. Another very great advantage of this system is that it diminishes the danger of interrupting an endogenous gene. For Plasmodium species this is an even greater advantage since Plasmodium genome is haploid for most of its life stages. When an intramolecular recombination occurs a specific DNA region located between two loxP sites can either be excised when the loxP sites are oriented the same or inverted when the loxP sites are in opposite orientation. This can be very helpful in cases when the selective marker needs to be removes so that it can be re- used for future approaches.

For introducing this system to an organism a vector that expresses the Cre recombinase is used. To make this system inducible and controllable several different methods have been developed. A classical one is to introduce a vector to the organism under study that carries the Cre recombinase gene under the control of a time or tissue specific promoter so that recombination occurs only at that specific time when the promoter is active or at that specific tissue where the promoter is working. Another approach is to fuse the Cre recombinase protein with a hormone binding domain, usually that of progesterone or estrogen steroid receptor. When the steroid is absent the receptor is kept in the cytoplasm by HSP (heat shock proteins) and so is the Cre recombinase. It is only after the addition of the steroid that the Cre recombinase/ steroid receptor complex can dissociate and the recombinase can enter the nucleus and facilitate recombination. It has though been proved that this does not work in *Toxoplasma gondii* since this species does not possess HSPs.

Another very evolutional approach of using the Cre mediated recombination is the DiCre system. For this the Cre protein is divided in two parts. The one is fused with one FKBP12 region and the other one with a FRB domain so that the two Cre parts cannot be linked without adding rapamycin. This system is inducible and is regulated by the concentration of rapamycin added. Moreover, every part of the Cre protein can be posed under the control of a tissue specific promoter so that recombination will occur only in that tissue where both promoters are operating.

FLP recombinase/ FRT system [33, 34]

This conditional mutagenesis system resembles the Cre/ loxP system to a great extent. Specifically, the FLP/FRT system originates from yeast, whereas Cre/loxP system is specific to P1 bacteriophages. It has been proved that this system works relatively well in both *Plasmodium falciparum* and *Plasmodium berghei*. It is though a problem that only blood stages can be genetically modified in Plasmodium species because only at that stages parasites are produced in large numbers required for electroporation techniques.

In this system the enzyme, which is the FLP (flip) recombinase, has the ability to specifically excise DNA through intramolecular recombination or to insert DNA through intermolecular

recombination, although this is kinetically less favorable than excision. It can also invert DNA located between two FRT sites with opposite orientation and exchange DNA from different chromosomes located upstream of the FRT sites. The FRT sites are homologous sites of a length of 34bp. This conditional mutagenesis system has been used a lot for conditionally knocking down genes that are essential for blood stages, so that the possible later role of these genes could be identified. This takes place through putting the FLP recombinase gene under the control of a time specific promoter. At the specific time point at which the promoter is active the FLP recombinase will be expressed and the sequence flanked with the FRT sites will be excised. It is though required to first exchange the endogenous gene of interest with another copy of the gene flanked with the FRT sites. This can be done either with single cross over recombination or double cross over recombination. Another very important tool for this system is the FLP-L recombinase, which is specifically active at lower temperatures with a high of activity at 21 °C, which is the temperature inside the mosquito. Therefore this enzyme is ideal for ensuring non excision in blood stages in the mouse and for maximum excision levels in mosquito stages.

Several approaches of how this system could be used for conditionally knocking down a gene have been tested. The idea is that a specific region of the gene of interest has to be flanked with FRT sites so that before recombination occurs the gene should be expressed normally and after recombination a total lack of the gene product should appear. Theoretically 5'UTRs, introns, exons and 3'UTRs could be flanked with FRT sites so that the gene would be knocked down. Practical, FTR flanking 5'UTRs did not yield profitable results. When introns or 3'UTRs regions were FRT flanked though the results were promising. There is also the possibility to FLP flank the whole gene, but that does not always work very well since the vector should not carry more than 1kb.

The great advantage of this system is that it does not require an exogenous added co factor. Despite that and although it works very well in Plasmodium species there appears to be a non-negligible leakiness. This is due to high stability of many proteins and mRNAs that are carried over from previous stages. Another reason is that a very high amount or enzyme is required because the FLP recombinase does not have a very high affinity for the FRT sites. Therefore developing the Cre/loxP system for Plasmodium seems very tempting, since Cre recombinase is very specific. It is though remarkable that under right conditions the FLP recombinase can reach 100%of activity, something that does not occur for the Cre recombinase.

Knock Sideway technique [35, 36]

KnockSideway technique is an innovating method of studying the function of a protein at a very specific time point in vivo. It could be characterized as a method of molecular dissection. This technique of conditional depletion of a protein has not yet been applied to Plasmodium species but it seems to work well in other eukaryotes.

For applying this technique to a cell line first the endogenous gene of interest must be knocked down by siRNA. Simultaneously a vector re-expressing a version of the endogenous gene tagged with a FKBP domain is transformed into the cell lines. This new protein will be refractory to depletion due to the FKBP linked domain. In the same cell line a mitotrapin gene linked to a FRB (FKBP rapamycin binding) domain is expressed. Mitotrapin is a protein that is anchored to the mitochondrion membrane. At the specific time point at which the role of the protein needs to be studied rapamycin is added to the cell. This results in binding of both the FKBP domain of the protein and the FRB domain of mitotrapin to rapamycin. Due to mitotrapin being anchored stably to the mitochondrion membrane, the protein is drugged away from the cytoplasm and sequesters to the mitochondrion membrane. This way the protein cannot complete its function and the depletion phenotype is achieved at a very specific time point at which the role of the protein can be easily studied.

KnockSideway techniques can also shed light on conjunction between proteins. To study if two specific proteins are linked one protein needs to be sequestered to the mitochondrion membrane. Every protein that follows depletion of this specific protein in cytoplasm and that is after depletion located at the mitochondrion membrane is in some way connected to the protein of study. It is a very quick and accurate way of understanding the functional links between different proteins.

This technique has been compared a lot to the siRNA technique. The great advantage of KnockSideway techniques is that it is rapid. Depletion is achieved within 5 minutes that is approximately 500 times quicker than depletion caused by siRNA. Due to KnockSideway techniques being very quick two significant problems caused by gradient depletion when using siRNA are circumvented. First, when a cell line experiences gradient loss of an essential protein it tries to find compensatory pathways of keeping the wanted phenotype and second when depletion is slow sometimes the protein might already have been expressed. Moreover, when using the siRNA technique it is not always clear if the depletion phenotype is caused due to scarcity of the protein at that specific time point or due to lack of this same protein at an earlier time point. Thus, this new technique of conditional depletion which is inducible can be very profitable for studying the role of a protein at a specific time point.

Tetracycline regulatable system [37]

This conditional knockdown technique has already successfully been applied to *Toxoplasma gondii*. Considering that Toxoplasma is closely related to Plasmodium, researches tried to transfer this same system to *Plasmodium falciparum*. Results showed that Tetracycline regulatable system does not work well but is though functional in *Plasmodium falciparum* and thus efforts are made to make it more effective so that it could give a powerful tool for studying the specific function of many haploid genes by conditionally knocking them down.

In order to develop that system the tetracycline repressor is fused to a transactivator and is so converted into an effective tetracycline controlled transactivator. For that a vector

carrying the tetracycline Repressor gene is transfected into the cells line of interest and by random integration of the Repressor gene into the genome of the parasite the tetracycline regulated transactivator is created. In the same cell line a TetO is inserted in front of the gene of interest. So once the tetracycline regulated transactivator tTA is expressed the repressor can bind to the TetO and the transactivator fused to the Repressor can activate the promoter in front of the Tet Operator. This happens only when the Repressor has the specific shape it obtains in absence of tetracycline. Once tetracycline is added the repressor changed shape and is thus no longer able to bind to the TetO. When that happens the transactivator cannot bind to the promoter of the gene and the gene is no longer expressed. An advantage of this system is that it is easily reversible by no longer adding tetracycline. It should also be mentioned that the system sometimes can also work in the opposite direction so that only when tetracycline is present the gene can be expressed.

This system has proved to be very fast in knocking down GFP, for which only a single cycle of life was required. In other cases though knocking down of a gene was not so fast. It is believed that the time the system needs to knockdown a specific gene is related to the stability of the mRNA, which can be carried over from previous stage. Further on, knocking down can also be delayed due to long life span of the protein deriving from the gene. That is why this system has not the same efficacy on all genes and that is considered as one of its disadvantages.

Another thing that should be taken into consideration is that this system has to be developed for each species individually. That is due to the trans-activators being very specific for each species. So the tetracycline regulatable system that has been applied to *Toxoplasma gondii* cannot be applied to mammalian cells, consistent with the fundamental differences in mechanisms in parasites and hosts. It is though possible to directly transfer the system developed In *Toxoplasma gondii* to *Plasmodium falciparum*, where it proved to be totally functional as mentioned previously.

Why to develop Tetracycline repressor aptamer system in *Plasmodium berghei*

The tetracycline repressor aptamer system is another conditional knockdown technique which has not yet been applied to any Plasmodium species. Researches have tried to apply this system to *Plasmodium falciparum* and until now results seem to be very promising tetracycline (J Nile group, MIT, USA - unpublished data). Since *Plasmodium falciparum* and *Plasmodium berghei* are very closely related and since the genome organization and the genetics are highly conserved between these two species it is very likely that the tetracycline repressor aptamer system will be functional in *Plasmodium berghei* as well.

The great advantage of this system is that it is regulatable. Expression of the protein of interest will be regulated upon tetracycline concentration. This means that if lower protein levels are required tetracycline of a lower concentration will be applied to the cell line.

What are aptamers and how do they regulate gene expression

It has been noticed that some regulatable systems that naturally occur in certain species make use of an aptamer-ligand system that seems to control some endogenous functions. $Gram^+$ bacteria tend to bind an effector to mRNAs to regulate gene expression, whereas in $Gram^-$ bacteria it has been noticed that certain leading sequences form a loop in response to an effector which overlaps with the start codon or the Shine Dalgarno regulatable sequence. Further on, mRNAs that encode enzymes involved in flavin mononucleotide or thiamine pyrophosphate biosynthesis bind their effectors FMN/TPP without using any trans- active proteins [38].

Aptamers could be characterized as riboswitches (molecular switches) that have the ability to sense their specific ligand with a great affinity [39] and bind it using a pocket construct and do so subsequently undergo restructuring. This new aptamer formation obtained upon ligand binding is sometimes a reinforced form that is refractory to melting ribosomes perform in order to unwind RNA and to start translation [40]. It has also been noticed that the aptamer-ligand structure might mask regulatory sites present in mRNA or alternately become an obstacle for binding or movement of ribosomes. Further on, transcriptional attenuation or ribosome-mediated mRNA degradation might occur upon binding [38]. This system seems to exploits great conformational flexibility and functional versatility.

The great advantage of this system is that aptamers respond to a dose dependent way to small non-metabolic and non-toxic molecules and the novelty that it introduces is that no trans activation events depending on a protein are required since the system relies totally on an RNA-RNA interaction. Riboswitches fulfill both sensory and regulatory functions in this case [37]. Making use of a non-cytotoxic, cell permeable and non-metabolic metabolite as a ligand which binds specifically to an aptamer could generate a very reliable conditional gene knockdown system. Tetracycline seems to be ideal for that purpose since it enters nearly all cells including passage of the blood brain barrier and placenta, is non-metabolic and can easily be monitored via its fluorescence and absorption profile. Unfortunately tetracycline has been proved to be toxic to *Toxoplasma gondii* and *Plasmodium berghei* and thus some tetracycline analogues (anhydrotetracycline, doxycycline) were used alternatively[37].

Some aptamer related properties have to be taken into consideration when trying to establish an aptamer dependent system for a specific species. Aptamer functionality is very delicate and depends upon many characteristics. It has been shown that aptamer regulatory properties can depend upon the site of insert, the potential to form secondary structures, thermodynamic stability of the aptamer and sequence variations [41].

ISP1 and ISP3 genes in Plasmodium species

All Apicomplexa, including Plasmodium, possess a pellicle which is composed of a surface plasmalemma and a double layered inner membrane complex (IMC). In the space between the plasmalemma and the IMC an actomyosin motor is located which is responsible for invasion, motility and replication of the parasites. In Plasmodium species the formation of the IMC is essential for all morphological changes which the parasite undergoes during its life cycle [43].

ISPs (IMC sub compartment proteins) show to have an apical localization and an IMC like pattern during sexual development, suggesting that they are associated to the IMC. It has been showed that they play a significant role in formation of the apical end of the cell during both sexual and asexual developmental stages. These proteins are localized at the anterior apical end of the parasite and contribute in defining polarity during zygote/ ookinete development. It has been shown that ISP genes are expressed at all developmental stages, and especially at the invasive stages (merozoites, sporozoites, ookinetes). Plasmodium species do contain only ISP1 and ISP3 genes. ISP1 is highly expressed in activated and inactivated gametocytes in both *Plasmodium falciparum* and *Plasmodium berghei*, showing an essential role in sexual development. It has been shown though that ISP1 is also expressed at lower levels in asexual stages, proposing that this gene might play a role in blood stage development as well [43].

It has been noticed that in all invasive stages of *Plasmodium berghei* ISP1 is located at the periphery. The observation that ISP1 is related to the IMC is patronized by the fact that ISP1 is myristoylated. Myrisoylation is a co-post-translational protein modification that is essential for membrane targeting and signal transduction. Further on ISP1 and ISP3 are partially co localized with a- tubulin and GAP45, which have been shown to be related to the IMC. Nonetheless, when taking a closer look it was shown that only ISP3 was associated with the meshwork- which is associated to the IMC- whereas ISP1 was found only in the soluble fractions. Further on in ISP3 knockdowns ISP1 is no longer restricted to the periphery but dispensed throughout the cytosol. The conclusion which could be drawn from these is that ISP3 is responsible for restricting ISP1 to the IMC [43].

ISP2 and ISP4 genes in Apicomplexa species have most probably derived from duplications of ISP1 gene in a common ancestor of Apicomplexa, the Coccidian. Theileria, which have lost ISP3 gene over the years, have been showed to have a different invasion method than other Apicomplexa. This strongly suggests that ISP genes are involved in invasive patterns. Plasmodium species have lost ISP2 and ISP4 and it has been noticed that ISP1 knockdown parasites did not survive. So it is very likely that ISP1 gene might have become essential to Plasmodium species through evolutionary pressure since Plasmodium has lost functional redundancy when losing ISP2 and ISP4. ISP3 knockdown parasites on the other hand were able to complete their whole life cycle indicating that ISP3 is not essential for survival. Despite the fact that only ISP1 has proved essential for parasites' development, both ISP1 and ISP3 are needed for apical end polarity during sexual and asexual development as their localization indicates [43].

The synthesis and organization of the pellicle and the apical complex are very delicate procedures and any proteins involved in these could possibly be essential for the parasite's survival. Therefore the study of ISP genes, which are most likely involved in these procedures, could lead to new intervention strategies to successfully control malaria. Further on the IMC also plays a significant role in parasites' differentiation. So taking into consideration that ISP genes are associated with the IMC the study of those genes could reveal their vital role for parasites in differentiation procedures as well [43].

Expression and function P25 protein family in Plasmodium species [44]

During asexual development of the Plasmodium parasites many surface proteins are synthesized de novo. P25 proteins are present on ookinete surface of all known *Plasmodium* species. P25 gene family encodes proteins essential for zygote development and mosquito midgut invasion and its transcription starts in asexual stages, but mRNAs get transnationally repressed by DOZI complex [42]. Expression of the P25 proteins is first detected about 2h after female gamete activation and fertilization [42].

Gene knockout experiments that have been performed for these proteins show that their expression is vital for survival of the parasite inside the unfriendly environment of the mosquito's midgut. Therefore this protein family has been used as a target for developing transmission blocking vaccines. Several transmission blocking P25 antibodies that have been developed show that ookinete movement inside the mosquito's midgut is considerably arrested/ slowed down in presence of these antibodies. Also transmission blocking activity is directly related to antibody levels used. Since ookinetes that are delayed in forming oocysts are not likely to survive in the vertebrate's host midgut due to the harsh proteolytic environment, transmission is successfully blocked. Therefore P25 proteins are important vaccine candidates against malaria.

Expression and function of HSP70 proteins in Plasmodium species [45]

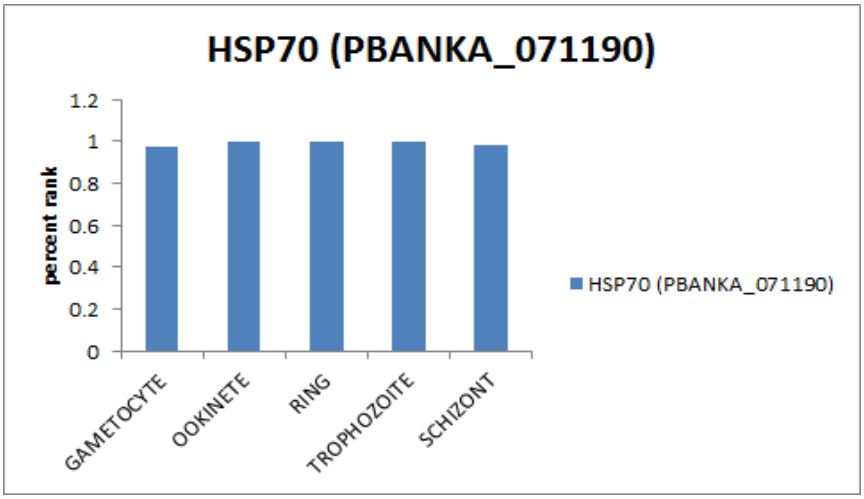
Heat Shock Proteins 70 (HSP70) are ATPases that can tightly bind peptides in their ADP-bound state and do so protect the polypeptide substrate from misfolding or aggregation. Therefore they are also referred to as chaperones. It has been shown that *Plasmodium* species encode at least four conserved HSP70 members that most likely fulfil chaperone functions and are localized in different compartments inside the parasite.

HSP70 members are ubiquitous and abundant in all life stages of *P. berghei.* Further on, *pb*HSP70/1 has been identified as the protein showing the highest expression levels during blood, mosquito and liver infection. In sporozoites weak expression of *pb*HSP70/1 was noticed, whereas expression analysis using a monoclonal antibody proved considerable up-

regulation in early liver stage development. These results give further evidence that *pb*HSP70/1 is constitutively expressed throughout the whole life cycle of *P. berghei.*

The exact function of *pb*HSP70/1 has not yet been revealed, but studies in *E. coli* and *S. cerevisiae* suggest that the *P. falciparum* analogue *pf*HSP70/1 is a major immunogen present in purified blood stage parasites.

All these findings together indicate that *HSP70/1* promoter is a strong candidate for transgenic expression of reporter proteins.



Expression profile of *HSP70* mRNAs in *P. berghei's* life stages (obtained from RNAseq A.P.W. database developed by Dr. Katie Hughes)

Scope of thesis

The aim of this thesis is to develop a novel conditional knockdown system for *Plasmodium berghei* that will allow researchers to study the dual role essential genes might fulfill in the latter sexual stages of the life cycle of the parasite. This system should circumvent several problems other conditional knockdown/out systems have brought up, such as leakiness or miss targeting of fusion proteins and should introduce certain advantages compared to other conditional knockdown-out systems.

Once developed this system should be inducible and totally time specific depending on the particular moment tetracycline will be provided. Another advantage should be its regulation upon tetracycline concentration as well as its reversibility after removal of tetracycline. Further on the target of this project is to develop a system that will have the potential to be easily applied for any gene researchers want to study.

Another goal of the present research is to develop two similar conditional knockdown systems that will function in the same way, only that the one will allow the study of the specific role of a gene throughout the whole life cycle of the parasite and the second the study of a gene only post activation of gametocytes.

The present study aims in releasing a novel conditional knockdown system for *Plasmodium berghei* that should have the potential to be easily transferred to other similar species, such as *Plasmodium falciparum,* in order to investigate roles and functions of genes that might be involved in transmission of malaria.

The aim of the secondary project- ISP project is to tag *ISP1* and *ISP3* proteins with a fluorescence protein called mCherry so as to be able to determine the specific position of these proteins throughout the whole life cycle of *Plasmodium berghei.* Investigating the position of these proteins, which are highly believed to be involved in forming of the apical complex of all invasive forms of the parasite (merozoites, ookinetes, sporozoites), will be very helpful in revealing their potential role in survival of the parasite.

3. Materials and methods

3a. Materials

All primers used for PCR amplifications and sequencing were custom synthesized from Eurofins MWG Biotech (see appendix). Taq polymerase was obtained from Invitrogen (Life Technologies) and Expand High Fidelity PCR system (HiFi Taq polymerase) was obtained from Roche. All restriction enzymes were acquired from New England Biolabs. Ligation of any insert into a plasmid was performed with a Roche rapid DNA ligation kit. *E.coli* cells used for transformation with a standard vector were Fusion- Blue Competent Cells K12 strain. For extraction of plasmid DNA Qiagen plasmid midi and mini preparation kits obtained from QIAGEN Sample & Assay Technologies were used. Gel extraction was performed using a QIAquick Gel Extraction. For transfections mice obtained from Harlan labs were used. Primary rabbit α-cMyc antibody purchased from Abcam, rabbit α-enolase was kindly donated from xxxx and α-actinI (PBANKA_145930) Ab was raised against a peptide (GNVKAGVAGDDAPRS) were obtained from Proteintech and secondary goat anti-rabbit-IgG was obtained from Dako.

3b. Methods- Experimental procedures

3b.1) Generation of vector plasmids

a) **Polymerase chain reactions (PCRs)**

Four kinds of PCRs were performed.

For all **genes which were PCR amplified using a DNA template and for all fragments larger than 3kb which had to be PCR amplified** Expand High Fertility PCR system from Roche was used.

Example: PCR reaction to amplify *tetr* using as template *pG323* (H.Patil- Waters Group)

PCR reaction

Template	1ul (100ng/ul)
dNTPs	1ul (0.2mM)
Primer 1 (GU2668)	1.5ul (0.3pmol/ul)
Primer 2 (GU2669)	1.5ul (0.3pmol/ul)
HiFi Taq	0.75ul (0.0525U/ul)
HiFi reaction buffer	15.0ul (1X)
ddH_2O	39.25ul
Total	50ul

PCR program

Step	Temperature	Time
Initial denaturation	94^{0}C	2 minutes
10 cycles	94^{0}C	15 seconds
	45-68^{0}C	30 seconds
	68^{0}C	1 minute/kb
20 cycles	94^{0}C	15 seconds
	45-68^{0}C	30 seconds
	68^{0}C	1 minute/kb
Final extension	72^{0}C	7 minutes
Hold	10^{0}C	Forever
End		

Every time the melting temperature had to be modified according to the melting temperature of the primers used in every different PCR reaction. The extension time had to be modified as well according to the length of the DNA fragment that were amplified every time.

For **colony PCRs and diagnostic PCRs** Taq DNA polymerase (5U/µl)(Invitrogen), 10x PCR buffer (200mM Tris-HCL pH 8.4, 500mM KCL) (Invitrogen), 50mM $MgCl_2$ (Invitrogen), 10pM/ul primers (Eurofins MWG Biotech) were used.

Example: Diagnostic PCR for confirming 5'UTR integration in line *hsp70:2cmyc:tetr* (G946).

PCR reaction

Template (G946)	2ul (50ng/ul)
dNTPs	1ul (0.2mM)
Buffer	5ul (1X)
$MgCl_2$	1.25ul (1.25Mm)
Primer 1 (GU1681)	2ul (0.4pmol/ul)
Primer 2 (GU0052)	2ul (0.4pmol/ul)
Taq	0.25ul (0.025U/ul)
ddH_2O	36.5ul
Total	50ul

PCR program

STEP	TEMPERATURE	TIME
Initial denaturation	95^{0}C	3 minutes
30 cycles	95^{0}C	15-30 seconds
	45-68^{0}C	15-60 seconds
	68^{0}C	1minute/kb
Final extension	68^{0}C	5-10 minutes
Hold	4-10^{0}C	Forever
End		

For **joining two individual PCR amplified fragments** together a different procedure had to be followed. Initially the two individual fragments had to be PCR amplified using the HiFi PCR program described above. This resulted in PCR product A and PCR product B. The two individual PCR fragments were joined using a HiFi PCR reaction without adding additional primers.

Example: PCR reaction in order to amplify *isp1*.

For amplifying *isp1* two set of primers were used, two merging at the two ends of the gene and two merging in the middle. The two primers in the middle were used in order to introduce EcoR-V restriction site in the middle of i*sp1*. For PCR A primer set GU2864/GU2867 (see Appendix) was used and for PCR B primer set GU2865/GU2866 (see Appendix) was used. PCR program and reaction mixture were the same as for the HiFi PCR reaction.

For the next PCR the template used was a mixture of PCR products A and B. For this step the HiFi PCR program was used with the melting temperature set slightly down so that fragments A and B could combine. Primers were not added to the reaction.

PCR reaction

Template	**1ul A+ 1ul B (100ng/ul)**
dNTPs	1ul (0.2mM)
Reaction buffer 10x	5ul (1X)
Taq polymerase	0.75ul (0.0525U/ul)
ddH_2O	41.25ul
Total	50ul

PCR program

Step	**Temperature**	**Time**
	94^{0}C	2 minutes
10 cycles	94^{0}C	15 seconds
	47-50^0 C	30 seconds
	68^{0}C	1 minute/kb
Final extension	72^{0}C	7 minutes
Hold	10^{0}C	Forever
End		

For the final PCR primer set GU2864/GU2865 (see Appendix) and 1ul of taq DNA polymerase (0.0525U/ul of final volume) were added. PCR program which was used was again the HiFi program. This reaction resulted in the whole PCR amplified *isp1* with the EcoRV restriction site introduced in the middle.

Last PCR was used in order to **anneal two individual oligos**. For this the oligos were kept for 5min at 95 ^{o}C and reaction was let to cool down for 30min.

b) **Molecular cloning procedure**

For construction of gene targeting plasmids, DNA inserts were PCR amplified using Expand High Fertility PCR system from Roche. PCR amplified DNA fragments were analysed through capillary separation on a 0.7% agarose/ TBE gel. All digestions of amplified DNA inserts and plasmid vectors were performed with New England Biolabs restriction enzymes and ligations were done using Roche rapid DNA ligation kit. Cloning of vectors occurred through heat shock transformation of *E.coli* cells. Qiagen plasmid midi or mini preparation kits were used for plasmid DNA extraction. Colony PCRs, diagnostic restriction enzyme digestions and sequencing (Eurofins MWG biotech) were performed to confirm the insert.

c) **Restriction enzyme Digestions**

For digestion of an insert or a vector prior to ligation 1µg of DNA, 2ul of restriction enzymes (20,000U), 4ul of 10x restriction buffer, 2ul of 50mg/ml BSA (ULTRA Pure^{TM} BSA obtained from Invitrogen life technologies), and ddH_2O up to 40ul were mixed and incubated for 1-2h at 37^{0}C.

For diagnostic digestions 1µg isolated plasmid DNA, 1ul restriction enzymes (20,000U), 2ul restriction buffer 10X and ddH_2O up to 20ul were mixed and incubated at 37^{0}C for 1-2h.

For digestions prior to transfections 5µg vector DNA, 10ul restriction enzymes (20,000U), 20ul restriction buffer 10X and ddH_2O up to 200ul were mixed and maintained at 37 ^{0}C for 2-3h. Additional 5ul restriction enzymes were added another two times and sample was again incubated for 2-3h at 37 ^{0}C.

d) **Ligation**

For ligation of an insert into a backbone vector, amount of insert DNA and vector DNA was calculated using ligation calculator- in silico. Rapid DNA Ligation Kit from Roche was used for all ligations.

Insert DNA and vector DNA were mixed (1 vector: 5 insert) with 1ul T4 DNA ligase (5U/ul), 10ul ligation buffer 2X and ddH_2O up to 20ul. Reaction was performed for 1h at room temperature.

e) **Generation of *2cmyc:tetr* containing plasmids**

The *tetracycline repressor (tetr)* was PCR amplified using pG323 (H. Patil- Waters group) as template. Forward primer GU2668 containing 2 *cmyc* in-frame just before start codon of *tetr* and reverse primer GU2669 were used. This amplified fragment was double digested with XhoI/XmaI and cloned into backbone vector pG0073 (K.Hughes-Waters group) opened with XhoI/XmaI, generating plasmid containing *5' UTRhsp70:2cmyc:tetr: 45/48 3' UTR* (pG366 also referred as *hsp70UTRs:2cmyc:tetr,* Figure A1). Insertion of *2cmyc:tetr* into the plasmid pG0073 was confirmed with colony PCRs and diagnostic digestions. Plasmid *hsp70UTRs:2cmyc:tetr* was sequenced to check mutations, linearized with SacII to be transfected into wild type (WT) *P. berghei* parasites targeted at P230P locus (PBANKA_030600)and should occur through a double cross over homologous recombination (Figure A2).

For constructing *P25 5'UTR:2cmyc:tetr:p25 3'UTR (*pG365 also referred as *p25:2cmyc:tetr,* Figure A3*)* the same procedure as for plasmid *hsp70UTRs:2cmyc:tetr* was repeated. This time pG0310 (H.Patil-Waters group), where the p25 promoter and p25 3'UTRs where already present, was used as backbone plasmid for insertion of *2cmyc:tetr* fragment. Linearized *p25:2cmyc:tetr* plasmid was transfected into wild type (WT) *P. berghei* parasites targeted at P230P locus (PBANKA_030600) and should occur by double cross over mechanism (Figure A4).

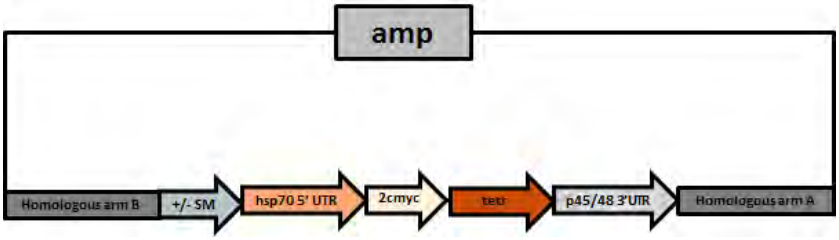


Figure A1: PCR amplified *2cmyc:tetr* cloned into backbone vector pG0073 generating *HSP70 5'UTR:2cmyc:tetr:P45/48 3'UTR* containing plasmid (pG366) (power point figure).

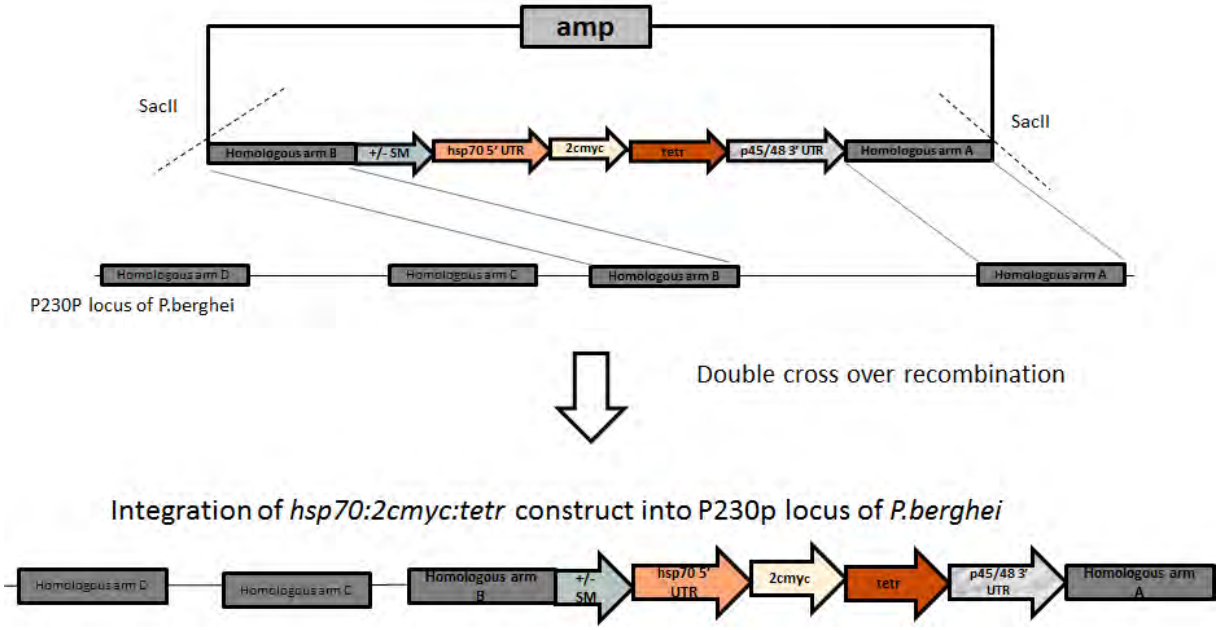


Figure A2: Plasmid *HSP70 5'UTR:2cmyc:tetr:P45/48 3'UTR (*pG366) was linearized with SacII to be transfected into wild type (WT) *P. berghei HPTBB* strain parasites, targeted at P230P locus (PBANKA_030600). Integration occurred through a double cross over recombination event.

Note: arms D and C are used for further transfection into this line

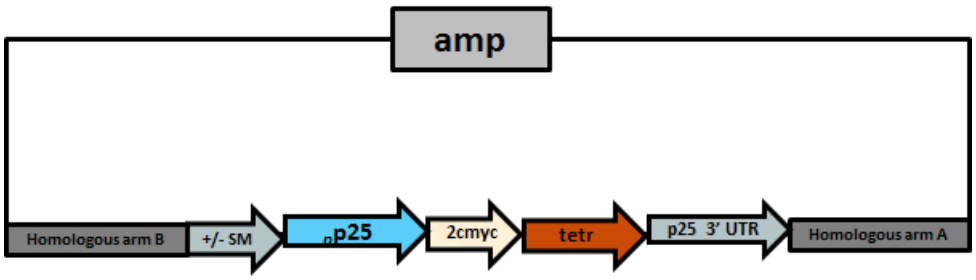


Figure A3: PCR amplified *2cmyc:tetr* was cloned into backbone plasmid pG310, generating *P25 5'UTR:2cmyc:tetr:P25 3'UTR* containing plasmid (pG365).

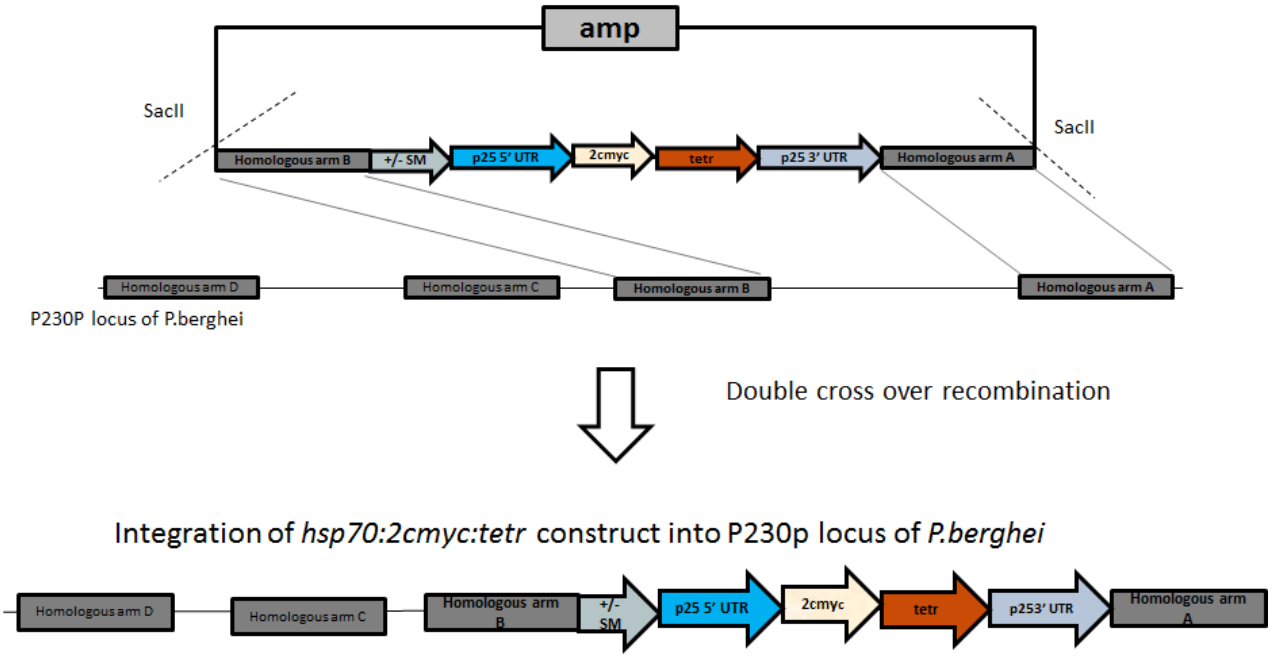


Figure A4: Plasmid *P25 5'UTR:2cmyc:tetr:P25 3'UTR (*pG365) was linearized with SacII to be transfected into wild type (WT) *P. berghei HPTBB* strain parasites targeted at P230P locus (PBANKA_030600). Integration occurred through a double cross over recombination event.

Note: arms D and C are used for further transfection into this line

f) **Generation of *functional aptamer: gfp* containing plasmid**

Functional aptamer sequence was obtained from J Nile group, MIT, USA (unpublished data).The same procedure as for generation *non-functional aptamer:gfp* containing plasmid was repeated (see below: **Generation of a *non-functional aptamer:gfp* containing vector**). Primers used for PCR amplifying *functional aptamer fragment* were forward primer GU2403 (containing the whole *functional aptamer* fragment- see Appendix) and reverse primer GU2402 (see Appendix). Sequencing results show a mutation- deletion of C (cytosine) at position 42 of the *functional aptamer* sequence (Figure B1). Therefore an alternative strategy was used.

Alternatively, two primer sets were used in order to amplify the *functional aptamer*. Annealing of the two oligos GU2985/GU2986 (see Appendix) by heating them at 95^0C for 5 min resulted in the 5' region of the *functional aptamer* (48bp). Similarly, annealing of oligos GU2987/GU2988 (see Appendix) resulted in the 3' region of the *functional aptamer* (49bp). The created 5' and 3' regions of *functional aptamer* were then combined resulting in the whole *functional aptamer (*97bp*)* (Figure B2). This fragment was digested with EcoRV/SalI and treated with Klenow (to create blunt ends) in order to be cloned into backbone vector pG394 (C.Manakanata- Waters group) digested with BsaBI, resulting in *pb eef1aa*

*5'UTR:functional aptamer:gfp:3' pb dhfr/ts (*also referred as *functional aptamer:gfp,* Figure B3). Insertion of *functional aptamer* was checked with colony PCR for transformed *E.coli* cells and diagnostic digestions. Plasmid *functional aptamer: gfp* was also sequenced to confirm the correct integration and to check mutations. Sequencing results again show different kind of mutations.

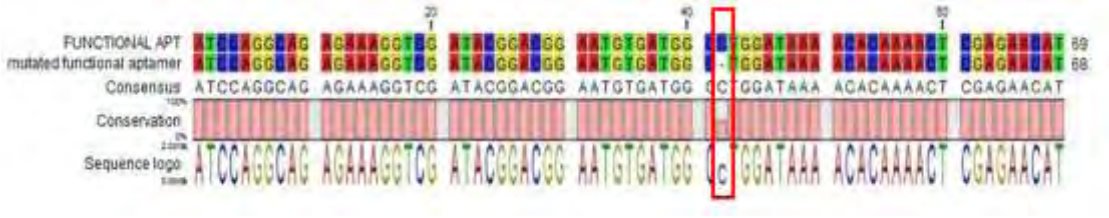


Figure B1: Sequencing results for *functional aptamer:gfp* containing vector show mutation-deletion of C (cytosine) at position 42 of *functional aptamer*.

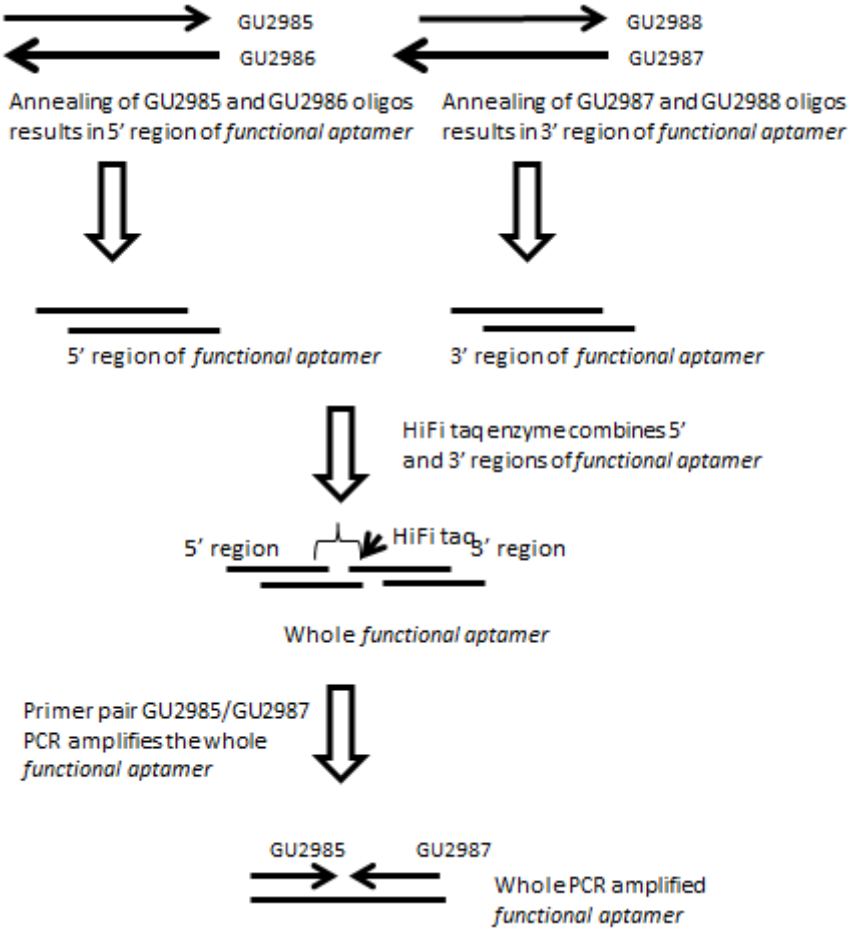


Figure B2: Oligo pairs GU2985/GU2986 and GU2988/GU2987 where annealed by being heated at 95 ^{0}C for 5min, resulting in 5' and 3' regions of *functional aptamer* respectively. These two fragments were combined into one by HiFi taq enzyme and finally primer pair GU2985/GU2987 was used to PCR amplify the whole *functional aptamer.*

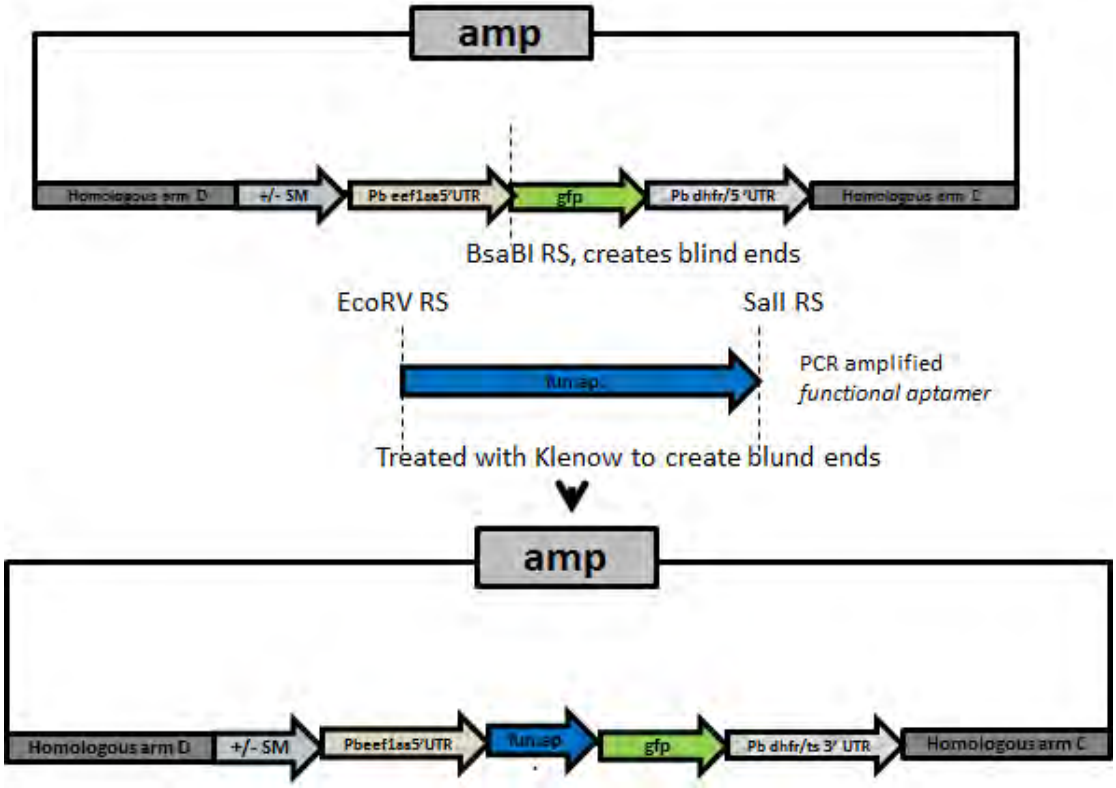


Figure B3: PCR amplified *functional aptamer* (see figure B2) was cloned into backbone vector pG394 (see Figure D1) resulting in *pb eef1aa 5'UTR:functional aptamer:gfp:pb dhfr/ts 3' UTR* containing plasmid.

g) **Generation of *non-functional aptamer: gfp* containing plasmid**

Backbone plasmid pL0015 (Waters group) was digested with KpnI/EcoRI to cut out the one homologous arm obtained from P230p locus of *P. berghei.* An alternative homologous arm obtained again from P230p locus of *P. berghei* (PBANKA_030600) was PCR amplified using WT genomic DNA of *HPTBB* strain as template and primer pair GU2862/GU2863 (see Appendix). This fragment was digested with KpnI/EcoRI and cloned into KpnI/EcoRI digested pL0015 resulting in pG213 (*pL0015: alternative P230p homologous arm C*, Figure C1). Insertion of the alternative homologous arm was checked with colony PCRs and diagnostic digestions. Backbone vector pG213 was also sequenced to rule out any mutations and to

confirm the integration and orientation of the insert. *Non-functional aptamer* was obtained from J Nile group, MIT, USA (unpublished data) and primer pair GU2404/GU2402 (see Appendix) was used in order to PCR amplify *gfp* using as template pL0031 (Waters Group) . Forward primer GU2404 (containing the whole *non-functional aptamer fragment*) introduced the *non-functional aptamer* in front of *gfp*. PCR amplified *non- functional aptamer:gfp* was digested with EcoRV/SmaII and cloned into BamHI digested/ Klenow treated (to create blunt ends) pG213, resulting in *pbeef1aa 5'UTR: non- functional aptamer:gfp:3'pb dhfr/ts* plasmid (pG379, also referred as *non-functional aptamer:gfp*, Figure C2). Insertion of *non-functional aptamer:gfp* was checked with colony PCRs and diagnostic digestions. Plasmid *non-functional aptamer:gfp* was sequenced to rule out any mutations and digested with EcoRI/SacII to linearize the construct for transfection into the P230p (PBANKA_030600) locus of WT *P. berghei HPTBB* strain(Figure C3).

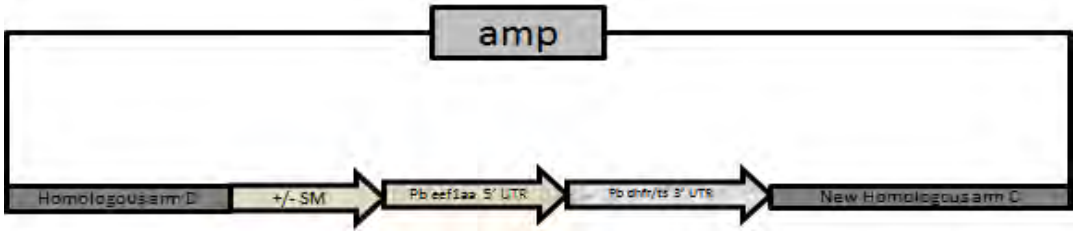


Figure C1: Primer pair GU2862/GU2863 was used in order to PCR amplify an alternative arm C obtained from P230p locus of *P. berghei* (PBANKA_030600)since the previous one present in backbone vector pL0015 overlaps with homologous arm B present in 2myc:*tetr* constructs.

Preexisting arm C was cut out with KpnI/EcoRI restriction enzymes and the alternative homologous arm C was cloned into backbone vector pL0015, resulting in *pL0015:new homologous arm C* plasmid (pG213).

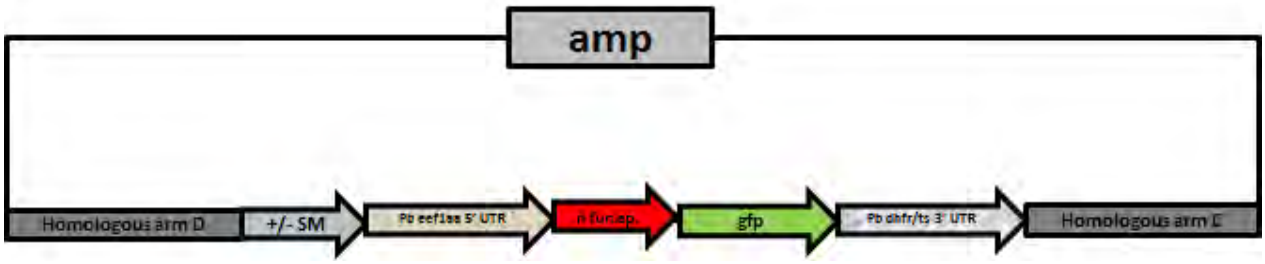


Figure C2: Primer pair GU2404/GU2402 was used to PCR amplify *non-functional aptamer:gfp* which was then cloned into backbone plasmid pG213 (see figure C1), resulting in *pb eef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTR* containing plasmid (pG379).

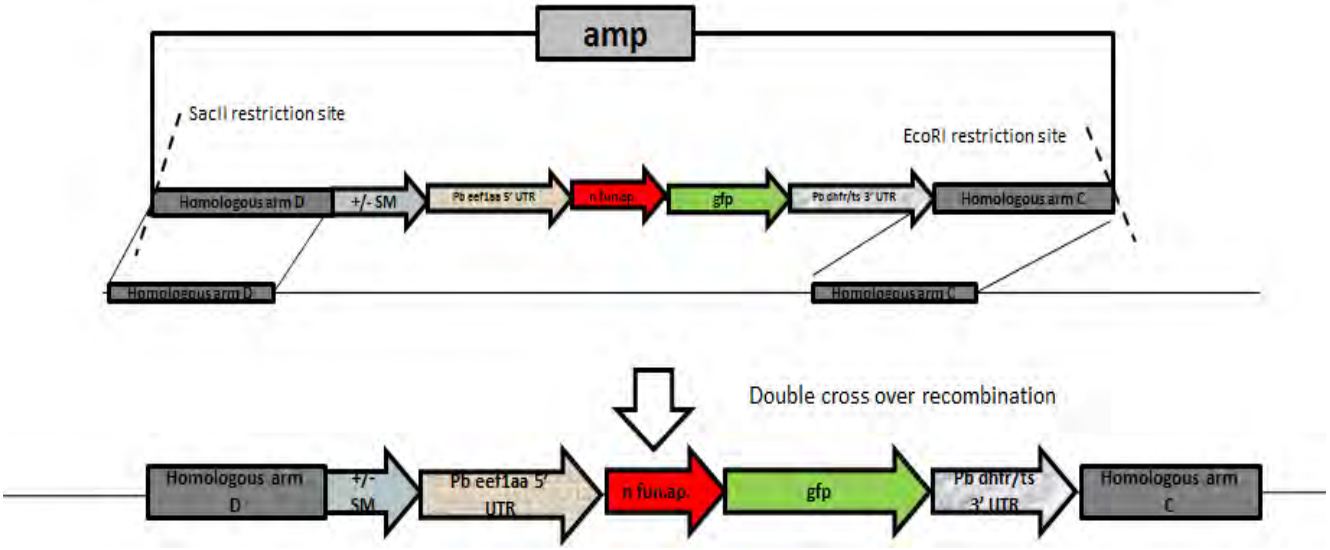


Figure C3: Plasmid *pb eef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTR* was linearized using EcoRI/SacII restriction enzymes and transferred into wild type (WT) *P. berghei HPTBB* strain parasites targeted at P230P locus (PBANKA_030600) through a double cross over recombination event.

h) **Generation of *gfp* containing plasmid**

The same procedure as for generating *non-functional aptamer:gfp* plasmid (pG379) was used. For PCR amplifying *gfp* primer pair GU2923/GU2402 (see Appendix) was used instead of GU2404/GU2402, since GU2923 does not introduce a *non- functional aptamer* sequence in front of *gfp*. PCR amplified *gfp* was digested with EcoRV/SmaII and cloned into the BamHI digested/ Klenow treated (to create blunt ends) pG213 resulting in *pbeef1aa 5'UTR:gfp:3'pb dhfr/ts* (pG394, Figure D1). This plasmid was then linearized with EcoRI/SacII restriction enzymes and transfection into the P230p (PBANKA_030600) locus of WT *P. berghei HPTBB* strain should occur through a double cross over recombination event (Figure D2).

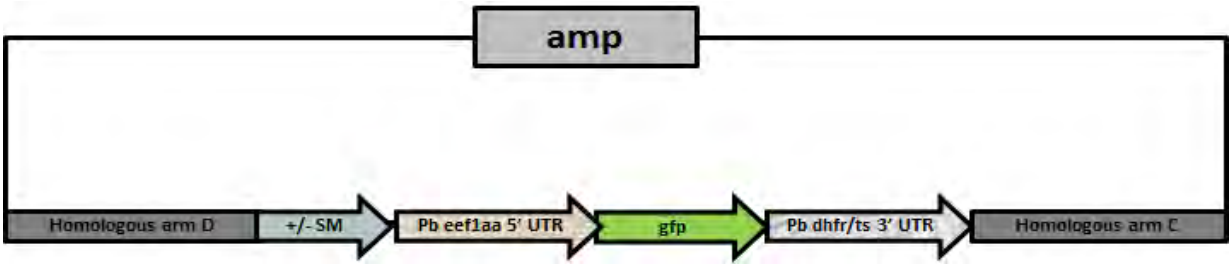


Figure D1: Primer pair GU2923/GU2402 was used in order to PCR amplify *gfp* which was cloned into backbone vector pG213 (see Figure C1), generating *pb eef1aa 5'UTR:gfp:pb dhfr/ts 3' UTR* containing plasmid (pG394).

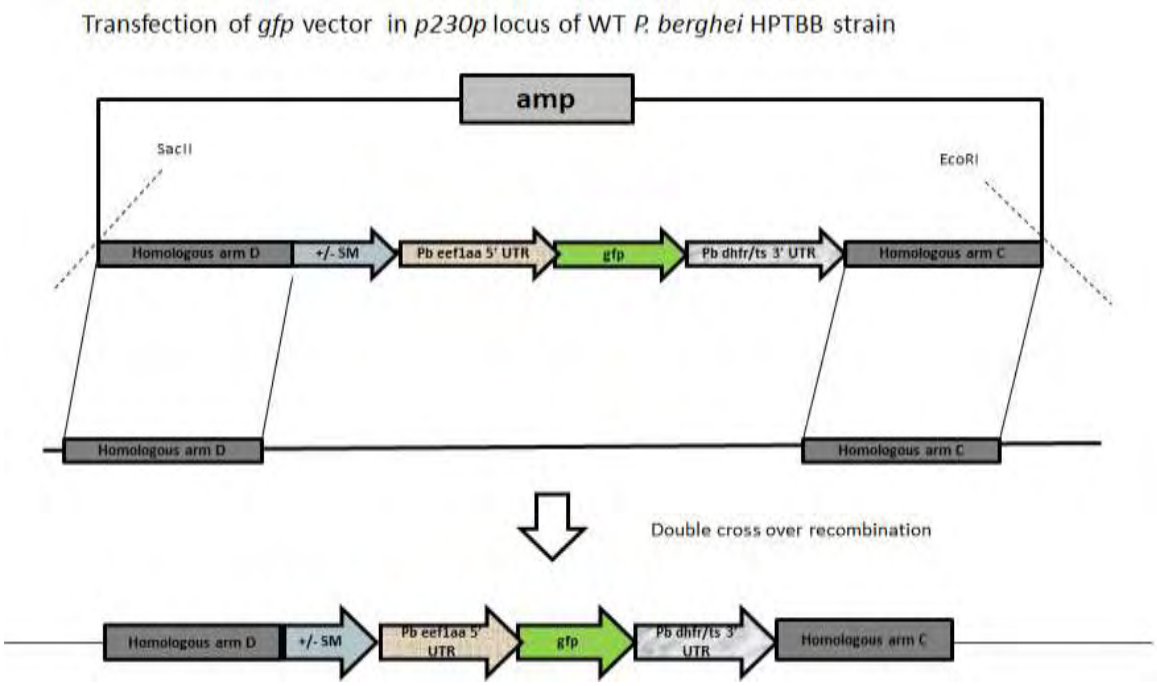


Figure D2: Plasmid *pb eef1aa 5'UTR:gfp:pb dhfr/ts 3' UTR* (pG394)was cut open using EcoRI/SacII restriction enzymes and transferred into wild type (WT*) P. berghei HPTBB* strain parasites targeted at P230P locus (PBANKA_030600) through a double cross over recombination event.

i) **Generation of *isp1* (PBANKA_120940*):mcherry* containing plasmid**

Primer sets GU2864/GU2867 and GU2865/GU2866 (see Appendix) were used to PCR amplify the 5' region (492bp) and the 3' region (608bp) of *isp1* respectively. Primers GU2866 and GU2867 were used to introduce an EcoRV restriction site in the middle of *isp1*. Reverse primer GU2865 was designed without a stop codon so that *isp1* could be tagged to *mcherry*. PCR amplified fragments 5' region and 3' region of *isp1* were combined into one single fragment (Figure E1). The whole constructed *isp1* was then digested with NotI/BamHI and cloned into NotI/BamHI digested pG267 (H.Patil- Waters group), resulting in *isp1:mcherry:3' pb 45/48* plasmid (pG393, also referred as *isp1:mcherry*, Figure E2). Insertion of *isp1* was first checked with colony PCR and diagnostic digestions and subsequently by sequencing *isp1:mcherry* plasmid (pG393). Plasmid *isp1:mcherry* (pG393) was then digested with EcoRV to linearize and transfected into *WT:green* male gametocytes strain targeted at 3' region of endogenous *isp1.* Integration should occur through a single cross over event (Figure E3).

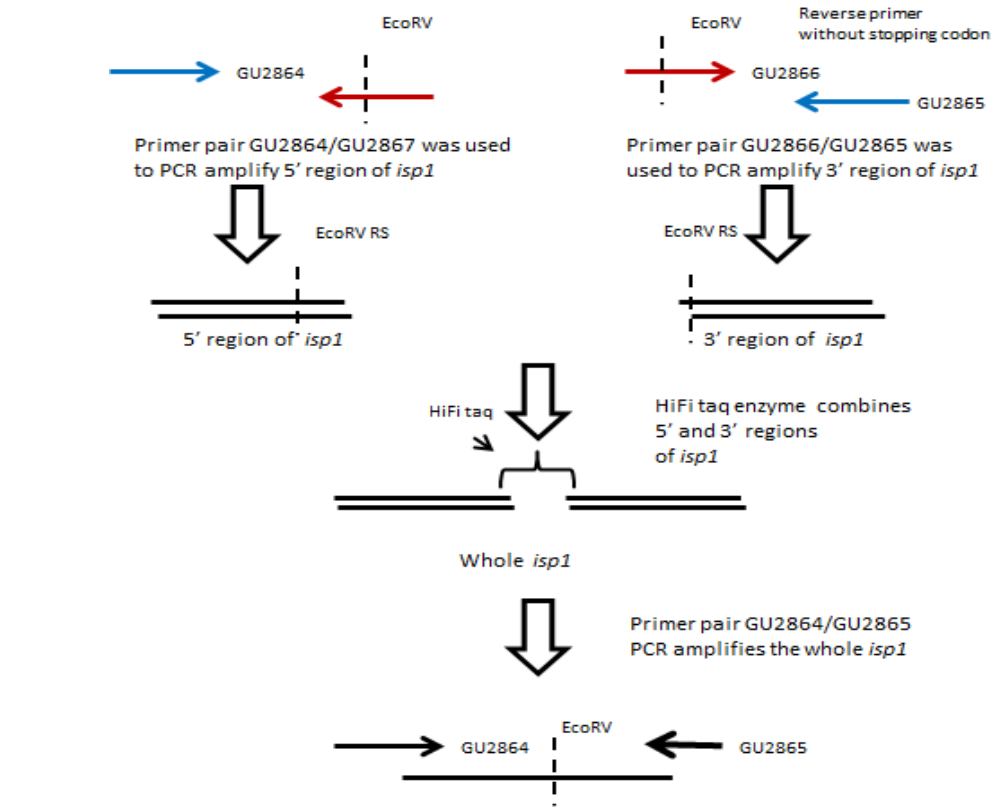


Figure E1: Primer pair GU2864/GU2867 and GU2866/GU2865 were used to PCR amplify 5' and 3' regions of *isp1* respectively. EcoRV restriction site was introduced in the middle of *isp1* by primers GU2866/GU2867. These two fragments were combined into one by HiFi taq enzyme and finally primer pair GU2864/GU2865 was used to PCR amplify the whole *isp1*.

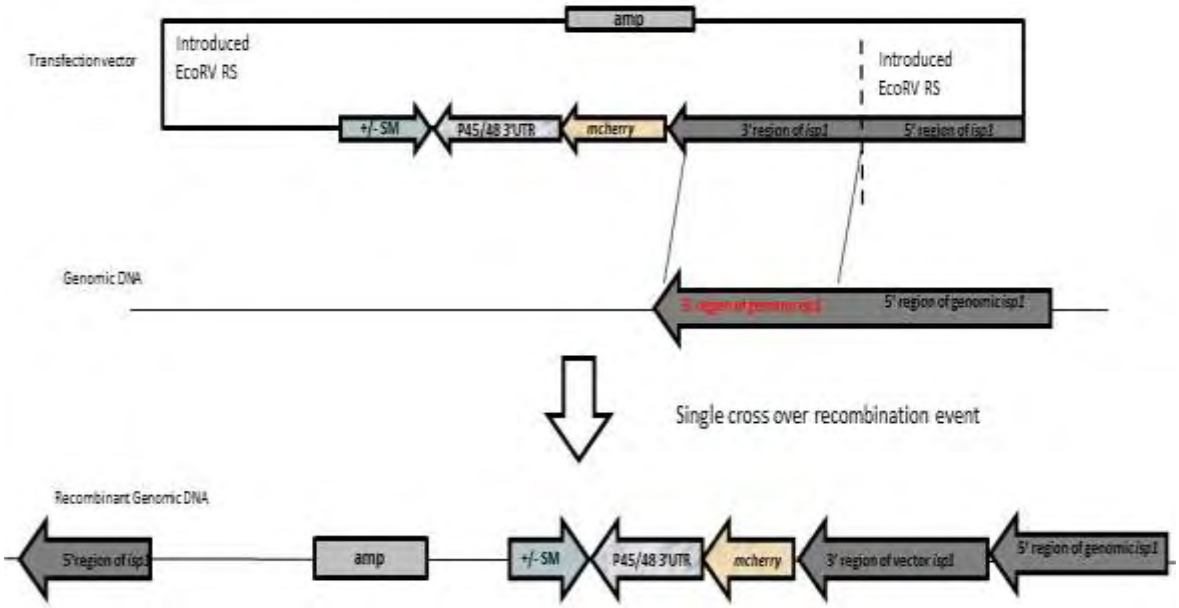


Figure E3: Vector *isp1:mcherry* cut open with EcoRV restriction enzyme was transferred into *WT:green* male gametocytes strain targeted at 3' region of endogenous *isp1.*

j) **Generation of a *isp3 (PBANKA_132430):MCherry* containing plasmid**

For generation of this plasmid the same procedure as for *isp1:mcherry* plasmid (pG393) was repeated (see Figure E1). Primer sets GU2921/GU2969 and GU2922/GU2970 (see Appendix) were used to PCR amplify the 5' region (575bp) and the 3' region (628bp) of *isp3* respectively. Forward primer GU2921 was designed 598bp upstream of the starting codon of *isp3* so as to obtain a fragment of about 1kb suitable for cloning into pG267 backbone vector. Reverse primer GU2922 was designed without a stop codon so that *isp3* could be tagged to *mcherry* and primer pair GU2969/GU2970 was used in order to introduce an EcoRV restriction site in the middle of *isp3*. PCR amplified *isp3* was digested with NotI/BamHI and cloned into NotI/BamHI digested pG267 (H.Patil- Waters group), resulting in *isp3:mcherry:3' pb 45/48* plasmid (pG399 also referred as *isp3:mcherry* , Figure F1).

Figure F1: PCR amplified *isp3* was cloned into backbone vector pG267 (H.Patil- Waters group) generating *isp3:mcherry:P45/48 3'UTR* containing plasmid (pG399).

3b.2) Transformation of *E. coli* cells with a standard vector

For transforming *E. coli* cells with a standard vector a mixture of the vector and *E. coli* cells (80ul) left for 10min on ice was heated at 42 °C for 45sec (heat shock transformation method). Transformed cells were then left to recover by first keeping them for 2min on ice and then by adding 250µl LB Broth dilution and maintaining them for 1h' at 37 °C in a shaking incubator. Transformed *E. coli* colonies were grown on an LB agar/ampicillin plate (0.1%) for 16-18h at 37 °C.

3b.3) Ethanol precipitation

For ethanol precipitating a vector prior to transfection, $1/10^{th}$ of DNA volume of NaAcetate and 2 times DNA volumes of 96% or 100% ethanol were added to DNA which was incubated at -20 °C for 2h or overnight. DNA was then spun at 4 °C for 30 minutes at 14,000 RCF using a table centrifuge and supernatant was removed. Two hundred ul of 70% ethanol were added to pellet DNA which was again spun at 4 °C for 5 minutes at 14,000 RCF and supernatant was removed. The remaining DNA pellet was spun for a last time at 4 °C for 1 minute at 14,000 RCF and supernatant was again removed. After DNA pellet had been left to air dry for 15-20min, it was diluted in 11.5µl ddH_2O and maintained at room temperature for 30min. In order to confirm DNA presence concentration was measured at 260nm.

3b.4) Preparation of glycerol stocks

For preparation of a glycerol stock of a prepared vector 1ml of overnight transformed bacteria culture was mixed with 200µl of sterile glycerol and maintained at -80 °C.

3b.5) Transfection procedure and positive selection

For obtaining linearized purified vector DNA, 25-50 µg of digested vector were fractionated on a 0.8% agarose gel and fragment not containing the ampicillin gene was gel extracted and ethanol precipitated. For transfection >5 µg of linearized purified DNA were added to blood stage schizont of WT *P. berghei* parasites of either HPTBB strain or 507tbb (constitutive expression of GFP under ef1α promoter). Transfection occurred through electroporation using an Amaxa nucleofector device (Lonza). Transfected parasites where then IV (intravenously) injected into an uninfected TO female mouse and 24h after transfection the infected animal was kept on pyrimethamine (7 mg/ml) which was provided in drinking water

so that positive selection due to integration of *dhfr,* which provides resistance to pyrimethamine (7mg/ml) to transfected parasites, could occur.

3b.6) Preparation of Giemsa slides and counting of parasitemia

In order to prepare a Giemsa slide for asexual parasite stages (blood sample obtained 48h after infection) and for gametocytes (blood sample obtained was kept on sulfadiazine for another 48h) tail blood sample (10ul) was added on a slide and smear was made by drugging another slide over the blood sample. The slide was left to dry for 1-2min and fixed with 70% methanol, which sticks erythrocytes to the glass slide and kills parasites. Slide was again left to dry for 2min and put in Giemsa for 15min after which the slide was washed with ddH_2O and left to dry. Using a light microscope parasitemia was counted as the percentage of infected red blood cells (iRBCs). Percent parasitemia was calculated as '(number of infected RBCs/number of total RBCs)* 100.

For schizonts and ookinete slide preparation 500ul of heart blood obtained from an infected mouse by cardiac puncture under anaesthesia mixed with schizont (37^{o} C for 24h) and ookinete (21^{o} C for 24h) media respectively were spun at 8,000rpm and 10ul of schizonts/ookinete resuspended pellet were added on the slide. The same procedure as for preparing Giemsa slides for asexual stages and gametocytes was followed.

3b.7) Cloning of transfectants by limiting dilution method

Parasitemia of the infected animal kept on pyrimethamine (7mg/ml) was counted until it reached 0.3-1%. At that time point blood obtained from the tail of this animal was diluted in PBS (1^{st} dilution). By counting RBCs on the hematocytometre under a light microscope and according to the counted parasitemia, number of infected red blood cells (iRBCs) present in 1ml was estimated. According to this several dilutions with PBS of the 1^{st} dilution were prepared so, that in each 200ul of final dilution 0.8 parasite was present (8 out of 10 uninfected mice used for cloning should get the parasites) and i.v. injected in each of the 10 uninfected animals used for cloning.

3b.8) Preparation of stabilates

For preparation of a stabilate of a cell line 1ml of blood was mixed with 1ml of PBS+ glycerol+ heparin (concentrations) and maintained in liquid nitrogen.

3b.9) Analysis of sexual stages of *P. berghei*

Infected animals (48h after infection) were treated with sulfadiazine (30µg/litre) added in drinking water for 48h in order to enrich for gametocytes, since asexual parasite stages are killed by the drug. Gametocytes were observed under the light microscope by preparing a Giemsa slide and gametocytomia was calculated as (number of male gametocytes + number of female gametocytes/ total RBC number)*100.

In order to analyse activation/exflaggelation centres 10ul heart blood obtained from an infected mouse (kept on sulfadiazine 48h after initial infection) by cardiac puncture under anaesthesia, was immediately added into 300ul ookinete culture medium (RPMI1640 (Sigma), R4130 containing 25mMHEPES and 2mM L-glutamine, 10mM Na2CO3, 5Uml^{-1} Penicillin, 5µgµl^{-1} streptomycin, 50µg ml^{-1} hypoxanthine, 50mM Xanthurenic acid, pH7.5-7.6, 20ml heat inactivated foetal bovine serum). Ten ul of this dilution were placed on a haemocytometer and after 15min exflaggelation centres were counted using the light microscope (10X or 40X lense).

For analysing ookinetes, 1ml heart blood sample blood obtained from an infected mouse (kept on sulfadiazine 48h after initial infection) by cardiac puncture under anaesthesia, was added into 30ml of ookinete culture medium and incubated for 24h at 21^{0}C. After this initial incubation ookinete development was observed by Giemsa staining of thin smears. The ookinete conversion rate was calculated as the estimated as the percentage of ookinetes/ ookinetes+ retorts+ sphericals. Visualization of ookinetes was achieved through tagging with p25 surface antibody.

3b.10) Preparation of fluorescence microscope slides

Blood sample (500ul) was spun at 8,000rpm and parasite pellet was resuspended in 1ml P25 antibody/PBS (1:5000). Blood sample was kept on the rotator for 15min and again spun. Parasite pellet was washed 3X in PBS and 500ul Hoechst due were added. Blood sample was spun a last time to remove supernatant. 10ul of the pellet were added on a slide.

3b.11) Preparation of asexual-blood stages and schizont parasite pellet

In order to obtain asexual-blood stages parasite pellet blood sample obtained from an infected mouse 48h after infection (parasitemia should be around 3%) by cardiac puncture under anaesthesia was mixed with 30-40ml of erythrocyte lysis buffer 1X (10X stock solution: 1.5M NH_4C, 0.1M $KHCO_3$, 0.01M EDTA) and maintained on ice for 10-15min. Sample was subsequently spun at 1800rpm for 8min and supernatant was removed. Pellet was again resuspended in 1ml erythrocyte lysis buffer 1X (10X stock solution: 1.5M NH_4C, 0.1M $KHCO_3$,

0.01M EDTA), spun for 30sec- 1min at 8000rpm and supernatant was again removed. Parasite pellet was stored at -80 ^{0} C.

In order to obtain schizonts, 1ml heart blood obtained from an infected mouse (48h after infection; parasitemia around 3%) by cardiac puncture under anaesthesia was added into 30ml schizonts culture media and was gassed (5%CO_2, 5%O_2, 90%N_2) for 30sec-1min so as to simulate the environment inside the vertebrate host. Blood was then incubated for 16-18h at 37^{0}C under shaking conditions (35rpm). This mixture was subsequently spun at 1800rpm for 8min and after removing the supernatant the same procedure as for preparation of asexual-blood stages parasite pellet was followed

3b.12) Preparation of gametocyte and zygote/ookinete pellet

For preparation of gametocyte parasite pellet, an animal was infected and 48h after infection (parasitemia at around 3%) kept on sulfadiazine for another 48h to enrich gametocytes. After obtaining 1ml blood by cardiac puncture under anaesthesia the same procedure as for preparing asexual/blood stages parasite pellet was used.

In order to prepare zygote/ookinete parasite pellet, an animal was infected and 48h after infection (parasitemia at around 3%) kept on sulfadiazine for another 48h to enrich gametocytes. 1ml blood sample obtained from this infected mouse by cardiac puncture under anaesthesia was added into ookinete media and maintained at 21 ^{o} C for 4h (when preparing zygote pellet) or 24h (when preparing ookinete pellet). This mixture was then spun at 1800rpm for 8min and after removing the supernatant the same procedure as for preparation of asexual-blood stages parasite pellet was followed.

3b.13) Genomic DNA isolation of malaria parasites

Prepared parasite pellet was resuspended in700ul TNE buffer (Tris pH 8.0, 5mM EDTA pH 8.0, 100 mMNaCl), 200ug RNase and 1% (v/v) SDS were added and total volume was adjusted to 1ml with H_2O. After incubating the mixture for 10min at 37 ^{0}C, 200ug Proteinase K were added and mixture was again incubated for 1h' at 37 ^{0} C. Next, buffered phenol, phenol: chloroform: isoamylalcohol (25: 24: 1) and chloroform: isoamylalcohol (24: 1) were added in three succeeding separated steps up to 1.5ml and after every addition the tupe was inverted several times and centrifuged for 5min at 14,000rpm. After every centrifugation only the upper aqueous phase was transferred to a new tupe. Isolated DNA was ethanol precipitated (as described above) and re-suspended in 300ul ddH_2O.

3b.14) Western Blotting procedure

For Western blotting analysis of asexual stages and gametocyte DNA parasite pellet was prepared immediately after obtaining blood sample from the infected animal. For analyzing schizonts DNA blood sample was added in schizont media and kept for 24h at 37^0C so that schizonts pellet could be prepared. For analyzing ookinete DNA using Western blotting blood sample was kept in ookinete media for 24h at 21 ^{0}C and parasite pellet was prepared. Proteins present in parasite pellets were lysed in Net2+ buffer (140 mM NaCl, 50 mM Tris [pH 7.4], 4mM Dithiothreitol, 0.01% Nonidet P-40,) supplemented with Protease inhibitor (Roche). Samples were mixed with 2X Laemmli sample buffer and subsequently separated on 10 or 12% SDS polyacrylamide gels so that they could be transferred onto a nitrocellulose membrane (Biosciences). After blocking the membrane with 5% milk/PBS-Tween (PBST), desired primary antibodies diluted in 5% milk/PBS-Tween (PBST) to adjust desired concentration were used for probing of plots. After washing blots 3 times with PBST for 10min each time, secondary antibodies (Polyclonal Goat α-Rabbit or α-mouse Ig/HRP-Dako 10000x) diluted in 5% milk/PBS-Tween (PBST) were used for reprobing blots. Signals were finally visualized by adding ECL solution (Thermo Scientific) on blots and exposing them to an X- ray film which was subsequently developed.

For probing blots with a different antibody exposed blots were stripped two times 5min each with 0.2M NaOH and reblocked in 5% milk/PBS-Tween.

3b.15) Negative selection

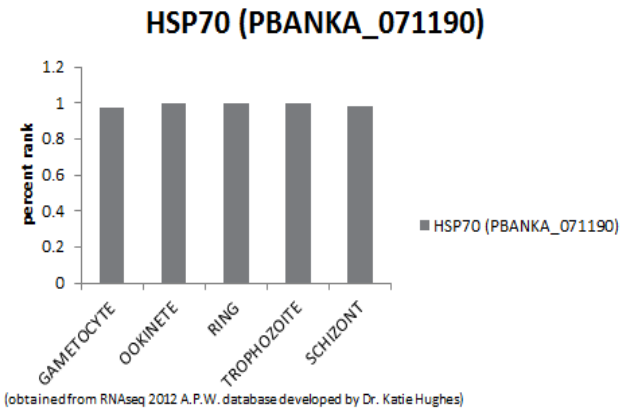
An animal was infected with a positively selected parasite line that contained a negative selection cassette integrated into its genome and was kept on pyrimethamine (7mg/ml) so that percentage of WT parasite would diminish further on. Once initial parasitemia had reached 0.3-1% (so that no multiple infected red blood cells would be present), the infected animal was kept on 5-fluorocytosine provided in its drinking water. After a strong decline of the initial parasitemia, once parasitemia had again reached 3-5%, the animal was bled and parasites were collected.

4. Results

4.a) Results for Tetracycline Repressor aptamer system project

4a.1) Generation of $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* parasite line (G946)

Introduction: Expression of Tetracycline repressor (*TetR*) was regulated by two different sets of promoters and 3'UTRs in two independent lines. Hsp70 promoter and p45/48 3'UTR which are known to be active throughout the life cycle of parasite [45] and P25 promoter and 3'UTR which are active only post activation of gametocytes in gametocytes and ookinete stages [44].



HSP70 (Heat Shock protein70 promoter) is highly active during the whole life cycle of *P. berghei* and therefore any gene set under its control will strongly be expressed in all life stages

Generation of *hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* parasite line (G946): Linearized plasmid *hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* (pG366) *(*digested with SacII restriction enzyme*)* was transfected into wide type (WT) *P. berghei* HPTBB strain. Integration of plasmid *hsp70 5'UTR:2cmyc:tetr:p45/48* (subsequently also referred to as $_{promoter}$*hsp70 5'UTR:2cmyc:tetr* vector) was checked by diagnostic PCRs at both (5' and 3') ends (using primers GU1681/GU2097 for 5', GU1682/GU2495 for 3'-see appendix)(Figure G1).

To confirm the expression of *2cMyc:TetR,* Western blotting analysis was performed on uncloned *hsp70 5'UTR:2cmyc:tetr* gametocytes and 4h zygotes using an α-cMyc (1:1000) antibody. Expected band size (26.2kD) for *TetR* was observed in uncloned $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* gametocytes and 4h zygotes(Figure G2). This is according to the Hsp70 promoter and p45/48 3'UTR activation profile, which are known to be active throughout the life cycle of the malaria parasite. The endogenously expressed protein enolase (concentration of antibody used was 1:3000) was used as a loading control.

After confirming stable integration of plasmid $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* at both ends and expression of *TetR* in gametocytes and 4h zygotes*,* uncloned $_{promoter}$*hsp70*

5'UTR:2cmyc:tetr:p45/48 3'UTRs (G946) line was cloned by limiting dilution. Seven animals were successfully infected and parasite DNA was checked by diagnostic PCRs at both (5' and 3') ends (Figure G3). Same primers as used previously for uncloned line $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* were utilized. Only 3 clones (subsequently referred to as Cl1, Cl2, Cl3 respectively) showed a possible 3' integration. Further diagnostic PCRs for confirming a stable 5' and 3' integration (using primers GU1681/GU0205 for 5', GU1681/GU2495 for 3'- see Appendix)(Figure G4) were performed and only Cl2 and Cl3 showed to be positive for a stable integration of both ends. Subsequently $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs*-clone 3 (Cl3) was chosen for further experimental procedures.

Growth defect testing experiments were performed for $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45 / 48 3'UTRs*-clone 3. Gametocytomia (Figure G5), exflagellation rates (Figure G6), ookinete conversion rates (Figure G7) and formation of ookinetes (Figure G8) was observed and was confirmed to be perfectly normal.

For $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs*- clone 3 Western blotting analysis using an α-cmyc (1:1000) antibody and Enolase/ActinI (1:3000) as a loading control was performed on asexual stages, schizonts, gametocytes and ookinetes (Figure G9) and expression of the *TetR* was confirmed in all stages.

Line $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs clone3* (subsequently also referred to as $_{promoter}$*hsp70:2cmyc:tetr-clone3*) was negatively selected to remove selectable marker by providing 5FC in drinking water (see methods for negative selection). Primers used for diagnostic PCRs in order to check if looping out of the SM was successful were GU1681/GU2496 for 5' and GU1682/GU2495 for 3' region (see Appendix)(Figures G10, G11).

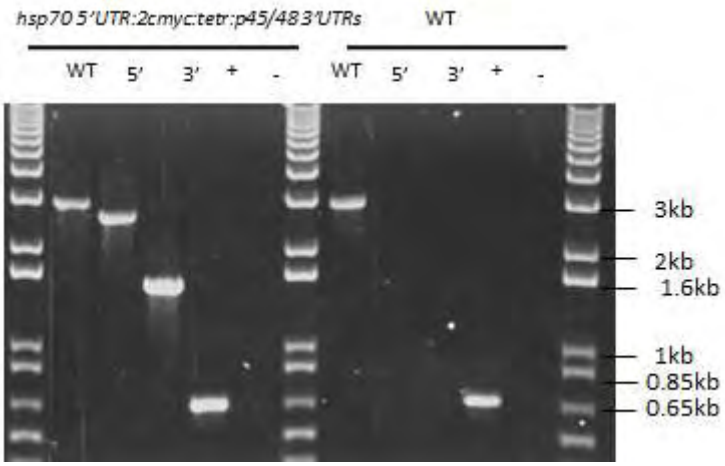


Figure G1: Uncloned line $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* (G946) shows appropriate genomic integration of both 5' (2626bp) and 3' (1713bp) ends into WT *P. berghei* genome. Presences of WT parasites (2800bp-WT, using primers GU1681/GU1682- see Appendix) was also checked and confirmed in both lines, as well as in WT, since lines are uncloned.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix) (700bp)

(-) Negative controls with no primer addition.

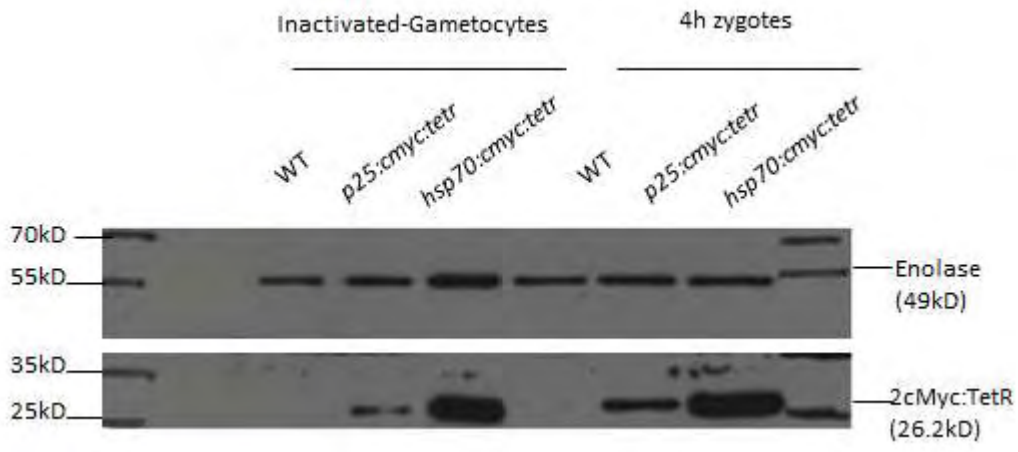


Figure G2: Western blotting analysis for lines $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* (G946) and $_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* (G945) indicated a stable expression of *TetR* in $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* line in both gametocytes and 4h zygotes and a stable expression of the *TetR* in line $_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* in 4h zygotes and only a partial expression in gametocytes.

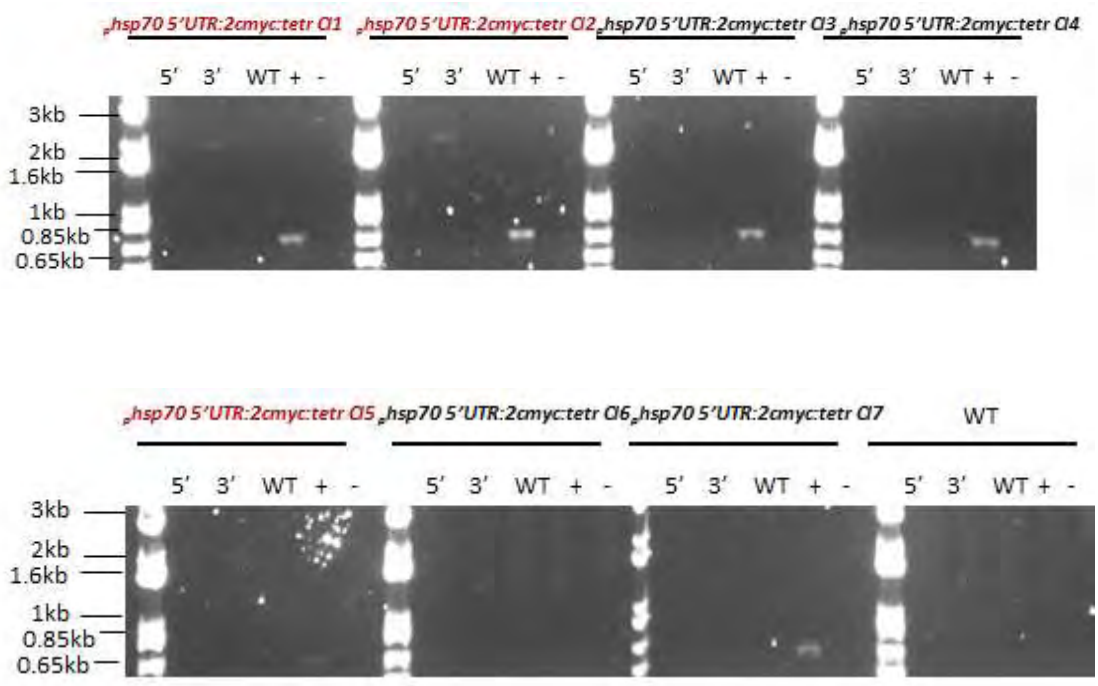


Figure G3: Line $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* (G946) was cloned and 7 positively infected animals were checked for an appropriate genomic integration of both 5'(2626bp) and 3' (1713bp)ends into WT *P. berghei* genome. Only 3 clones (subsequently referred to as Cl1, Cl2, Cl3 respectively) showed a possible 3' integration. Presences of WT parasites (2800bp- WT, using primers GU1681/GU1682-see appendix) was also checked and confirmed in WT only.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix) (700bp)

(-) Negative controls with no primers added.

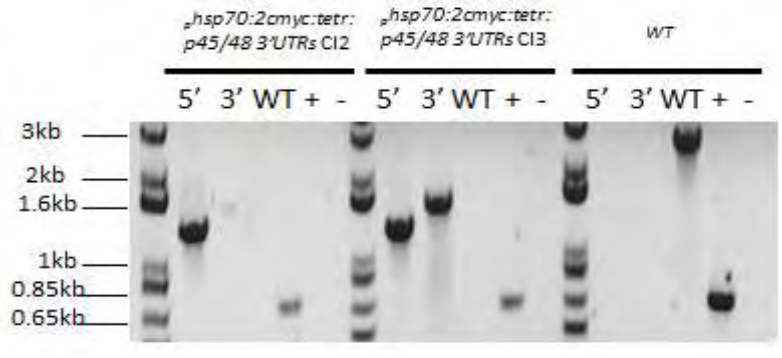


Figure G4: Diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr:p45/48 3' UTR*-clone 2 and *hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTR*-clone 3 lines show appropriate genomic integration of

both 5' (1384bp, using primers GU1681/GU0205-see Appendix) and 3' (1713bp, using primers GU1681/GU2495-see Appendix) ends into WT *P. berghei* genome. Presences of WT parasite (2800bp- WT, using primers GU1681/GU1682-see Appendix) was also checked and only confirmed in WT HPTBB parasite DNA so the lines were confirmed to be pure cloned lines.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix) (700bp)

(-) Negative controls with no primers added.

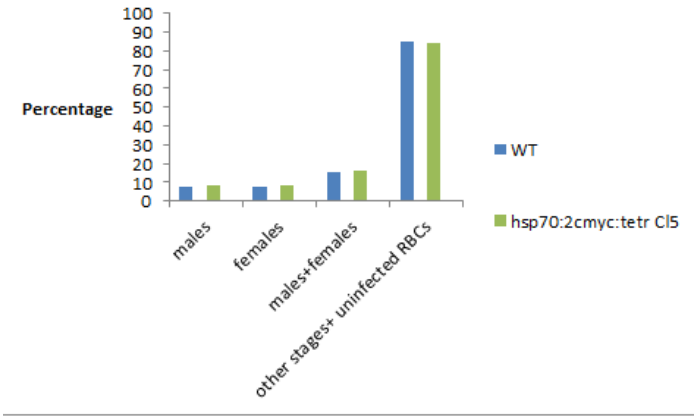


Figure G: Gametocytomia was counted for *hsp70:2cmyc:tetr* Cl5 line and was found to be at same levels as gametocytomia counted for WT HPTBB strain of *P. berghei.*

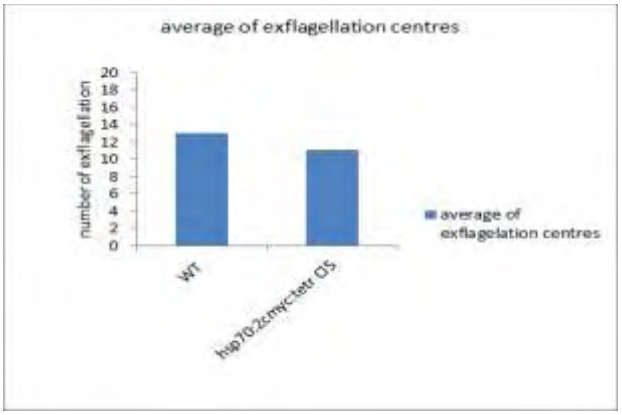


Figure G6: Exflaggelation/activation centres observed and counted under the light microscope for *hsp70:2cmyc:tetr* Cl3 are totally comperable to exflagellation centres counted in WT HPTBB *P. berghei* strain.

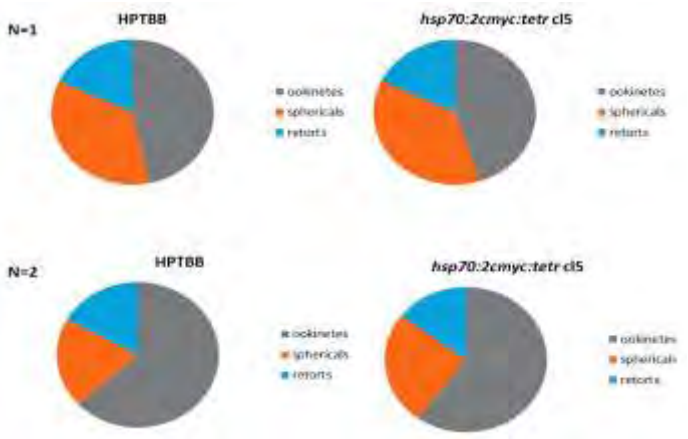


Figure G7: Ookinete conversion rates for *hsp70:2cmyc:tetr* Cl3 line seem to be normal when using WT HPTBB stain of *P. berghei* as a control.

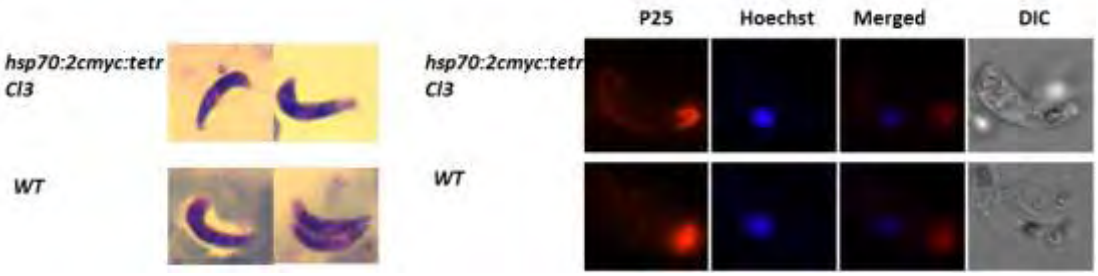


Figure G8: Ookinete formation for *hsp70:2cmyc:tetr* Cl3 seems to be perfectly normal.

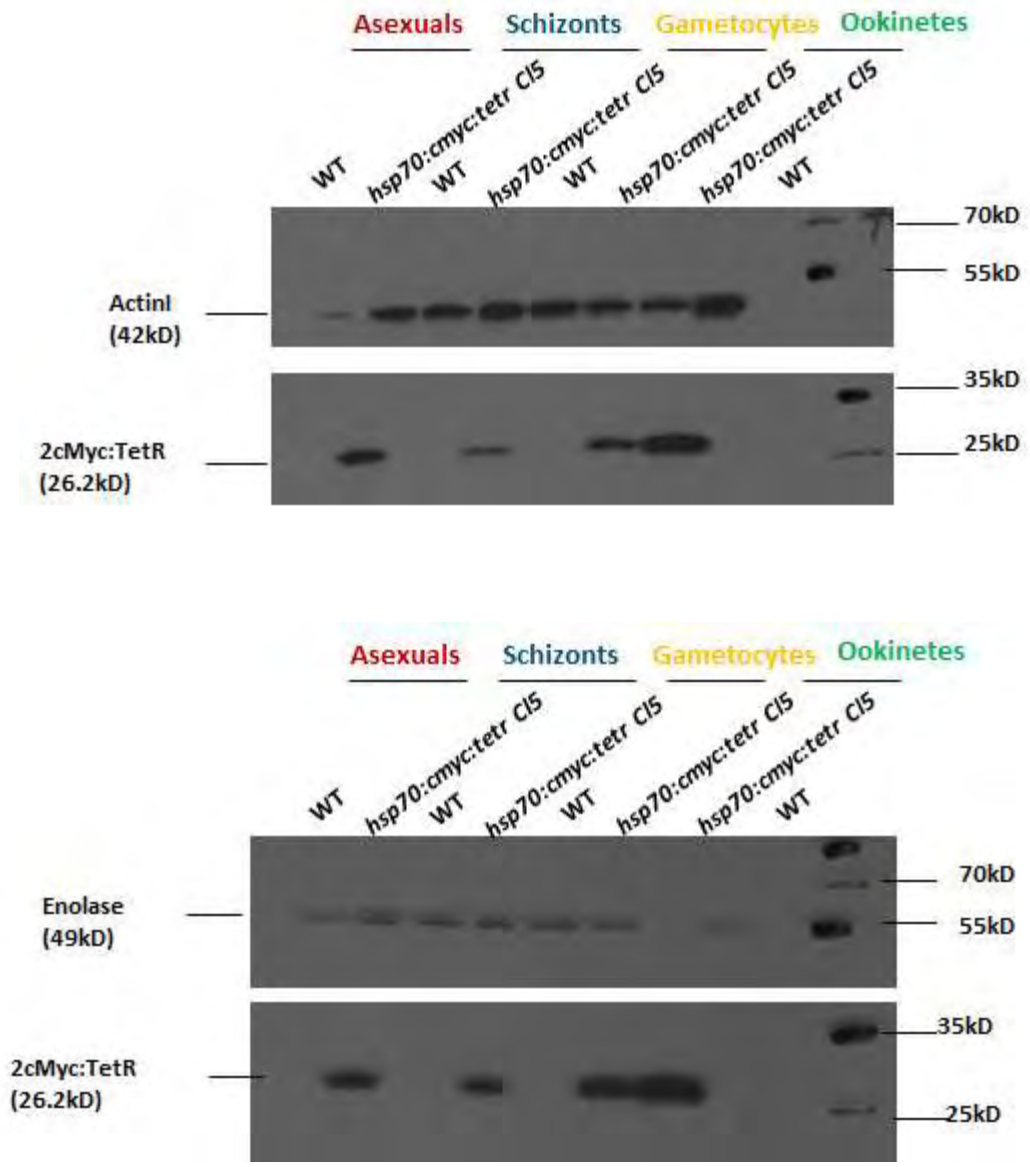


Figure G9: Western blotting analysis using an α-cmyc antibody confirmed expression of the *TetR* at most developmental stages. Endogenously expressed proteins ActinI and Enolase where used as loading controls.

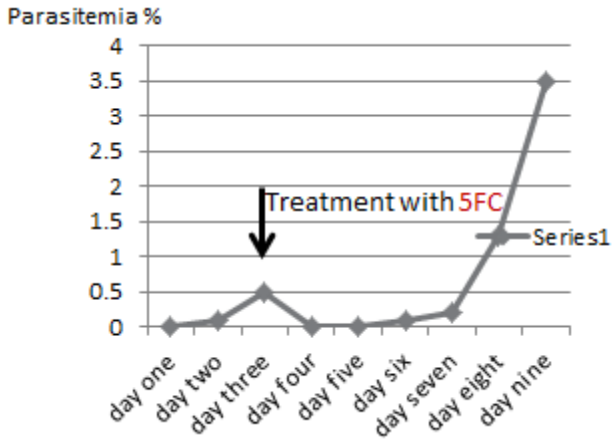


Figure G10: Animal infected with line *hsp70 5'UTR:2cmyc:tetr:p45/48 3' UTR*-clone 3 was kept on 5FC once initial parasitemia had reached 0.5%. After a short period during which strong declination of parasitemia was noticed, the percentage of parasites in blood raised again since only parasite that had successfully looped out the SM survived.

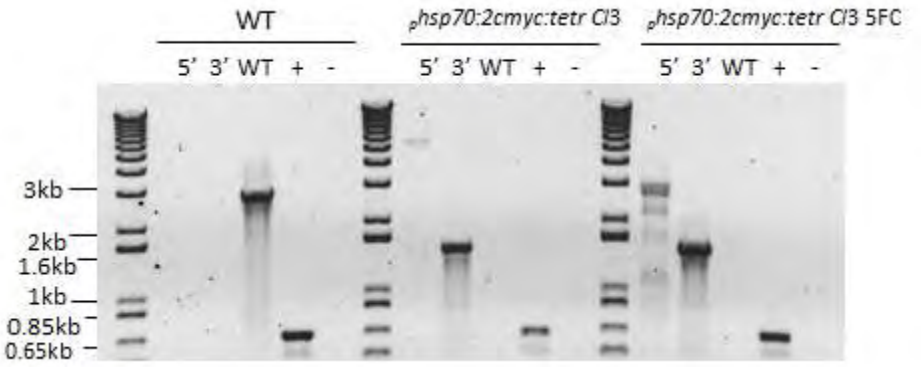


Figure G11: Line *hsp70 5'UTR:2cmyc:tetr:p45/48 3' UTR*-clone 3 was negatively selected. Results indicate that the selective marker was successfully looped out (5' Cl3: 6072bp, 5' Cl3 put on 5FC: 3200bp). Primers used for 5' integration checking were GU1681/GU2496 and primers used for confirming 3' integration were GU1681/2495 (see Appendix). Presences of WT parasite (2800bp- WT, using primers GU1681/GU1682-see Appendix) was also checked and only confirmed in WT HPTBB parasite DNA since this is a cloned line.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix) (700bp)

(-) Negative controls with no primers added.

4a.2) Generation of $_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* parasite line (G945)

Premade plasmid $_{promoter}$*p25:2cmyc:tetr:p25 3'UTRs* was linearized using SacII restriction enzyme and transfected into WT *P. berghei HPTBB* strain. Genomic integration of plasmid $_{promoter}$*p25:2cmyc:tetr:p25 3'UTRs (*subsequently also referred to as $_{promoter}$*p25:2cmyc:tetr)* was checked at 5' and 3' ends (using primers GU1681/GU2097 for 5', GU1682/GU2495 for 3'- see appendix) performing diagnostic PCRs. Integration of the whole *+/-SM:P25 5'UTR:2cmyc:tetr:P25 3' UTR* fragment was confirmed(Figure H1).

Western blot analysis was done to confirm the expression of *TetR* in $_{promoter}$*p25:2cmyc:tetr:p25 3'UTRs* inactivated gametocytes and 4h zygotes using α-cMyc antibody (Ab). Expression of *2cMyc:TetR* was confirmed in 4h zygotes, however, some *2cMyc:TetR* was also detected in inactivated gametocytes (figure G2). This indicates that gametocytes might have activated to a little extent at 21 ^{0}C room temperature or leaky expression of p25 UTRs in gametocytes.

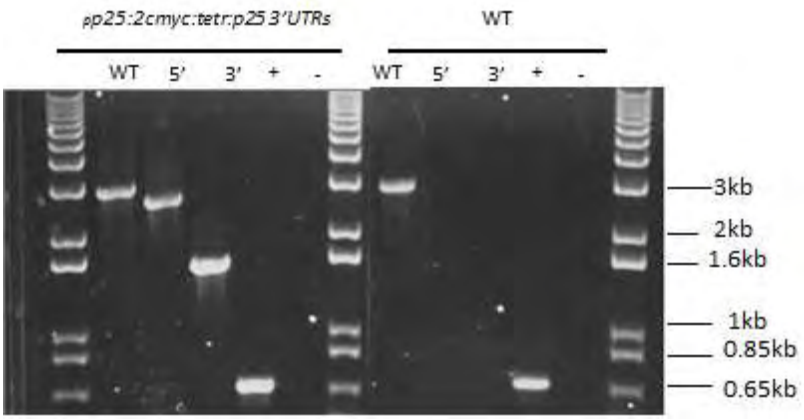


Figure H1: Uncloned line $_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* (G945) shows appropriate genomic integration of both 5' (2626bp) and 3' (1713bp) ends into WT *P. berghei* genome. Presences of WT parasite (2800bp-WT, using primers GU1681 / GU1682-see Appendix) was also checked and confirmed in WT parasites as well as in $_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* line since line is uncloned.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix) (700bp)

(-) Negative controls with no primer addition.

4a.3) Generation of Aptamer parasite line *P. beghei*

Introduction: It has been noticed that some regulatable systems that naturally occur in certain species make use of an aptamer-ligand system that seems to control some endogenous functions [36].

Aptamers could be characterized as riboswitches (molecular switches) that have the ability to sense their specific ligand with a great affinity[39] and bind it using a pocket construct and do so subsequently undergo restructuring. The great advantage of this system is that aptamers respond to a dose dependent way to small non-metabolic and non-toxic molecules and do so regulate gene expression [37].

Aptamer functionality is very delicate and depends upon many characteristics. It has been shown that aptamer regulatory properties can depend upon the site of insert, the potential to form secondary structures, thermodynamic stability of the aptamer and also sequence variations [41].

Generation of *non-functional aptamer* parasite line *P. berghei* (G967)*:* Linearized plasmid *$_{promoter}$pbeef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTRs* (subsequently also referred to as *non.fun.apt:gfp*) *(*digested with SacII/EcoRI restriction enzymes*)* was transfected into wilde type (WT) *P. berghei* HPTBB strain. Integration of plasmid *$_{promoter}$pbeef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTRs* was checked by diagnostic PCRs at both (5' and 3') ends (using primers GU1028/GU1485 and GU1028/GU0617 for 5', GU0161/GU2923 and GU0161/GU2438 for 3'- see appendix)(Figure I1).

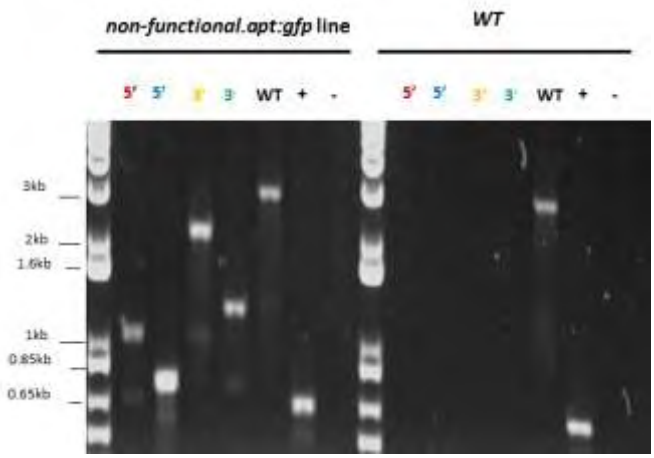


Figure I1: Uncloned line *$_{promoter}$pbeef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTRs (*G967*)* shows appropriate genomic integration of both 5' (5' 1059bp, 5' 5 774bp) and 3' (3' 2169bp, 3' 1259bp)ends into WT *P. berghei* genome. Presence of WT parasite (2978bp- WT, using primers GU1028/GU0161-see appendix) was also checked and confirmed in WT control as well as in line *$_{promoter}$pbeef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTRs (*G967*)* since line is uncloned.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix)(700bp)

(-) Negative controls with no primer addition.

4a.4) Generation of *gfp* expressing parasite line

Linearized plasmid $_{promoter}$*pbeef1aa 5'UTR:gfp:pb dhfr/ts 3'UTRs* (subsequently also referred to as *gfp* plasmid) *(*digested with SacII/EcoRI restriction enzymes*)* was transfected into wild type (WT) *P. berghei* HPTBB strain. Integration of plasmid $_{promoter}$*pbeef1aa 5'UTR:gfp:pb dhfr/ts 3'UTRs* was checked by diagnostic PCRs at both (5' and 3') ends (using primers GU1028/GU1485 and GU1028/GU0617 for 5', GU0161/GU2923 and GU0161/GU2438 for 3'-see appendix)(Figure J1).

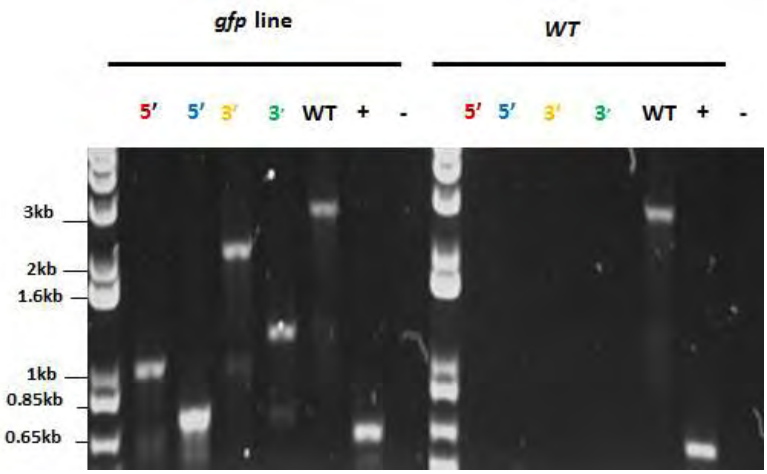


Figure J1: Uncloned line $_{promoter}$*pbeef1aa 5'UTR:gfp:pb dhfr/ts 3'UTRs (*G1005*)* shows appropriate genomic integration of both 5' (5' 997bp, 5' 714bp) and 3' (3' 2169bp, 3' 1259bp)ends into WT *P. berghei* genome. Presence of WT parasite (2978bp- WT, using primers GU1028/GU0161-see appendix) was also checked and confirmed in WT control as well as in line $_{promoter}$*pbeef1aa 5'UTR:gfp:pb dhfr/ts 3'UTRs (*G967*)* since line is uncloned.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix)(700bp)

(-) Negative controls with no primer addition.

4b) Results for ISP (IMC Subcompartment Proteins) project

4b.1) Generation of mcherry tagged *isp*1 parasite line.

Introduction: All Apicomplexa, including Plasmodium, possess a pellicle which is composed of a surface plasmalemma and a double layered inner membrane complex (IMC). In Plasmodium species the formation of the IMC is essential for all morphological changes which the parasite undergoes during its life cycle [43].

ISP (IMC subcompartment proteins) genes are specific to Apicomplexa species and are highly likely to be involved in invasion patterns [26]. ISPs (IMC sub compartment proteins) show to have an apical localization and an IMC like pattern during sexual development, suggesting that they are associated to the IMC.

The synthesis and organization of the pellicle and the apical complex are very delicate procedures and any proteins involved in these could possibly be essential for the parasite's survival. Therefore the study of ISP genes, which are most likely involved in these procedures, could lead to new intervention strategies to successfully control malaria.

Generation of *isp1:mcherry* expressing parasite line *P. berghei:* Linearized plasmid *isp1:mcherry:p45/48 3'UTRs* (subsequently also referred to as *isp1:mcherry* plasmid) (digested with EcoRV restriction enzyme) was transfected into *WT:green* male gametocytes strain targeted at 3' region of endogenous *isp1*. Integration of plasmid *isp1:mcherry:p45/48 3'UTRs* was checked by diagnostic PCRs at both (5' and 3') ends (using primers GU2989/GU1934 for 5', GU2990/GU2994 for 3'- see appendix)(Figure K1).

Expression of *ISP1* was checked with live fluorescence images and results showed that *ISP1* is expressed in gametocytes and 2,45h zygotes. Expression in zygotes shows to be higher than in gametocytes and also *ISP1* seems to be restricted to the plasma membrane rather than being distributed throughout the cytoplasm in zygotes (Figure K2).

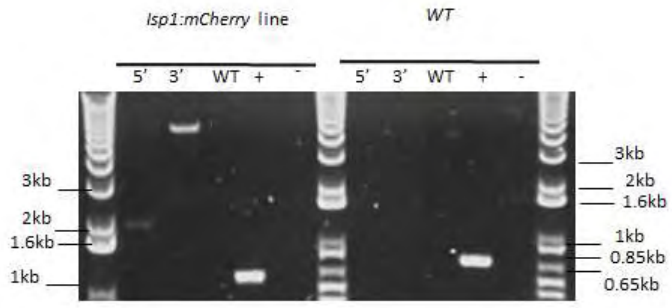


Figure K1: Uncloned line *isp1:mcherry:p45/48 3'UTRs* (G1004) shows appropriate genomic integration of both 5' (1896bp) and 3' (6843bp) ends into WT *P. berghei* genome. Presence of WT parasite (1000bp- WT, using primers GU2992/GU2989-see appendix) was also checked and not confirmed in WT control.

(+) Unrelated positive PCR controls using primer pair GU0013/GU0014 (see Appendix) (700bp)

(-) Negative controls with no primer addition.

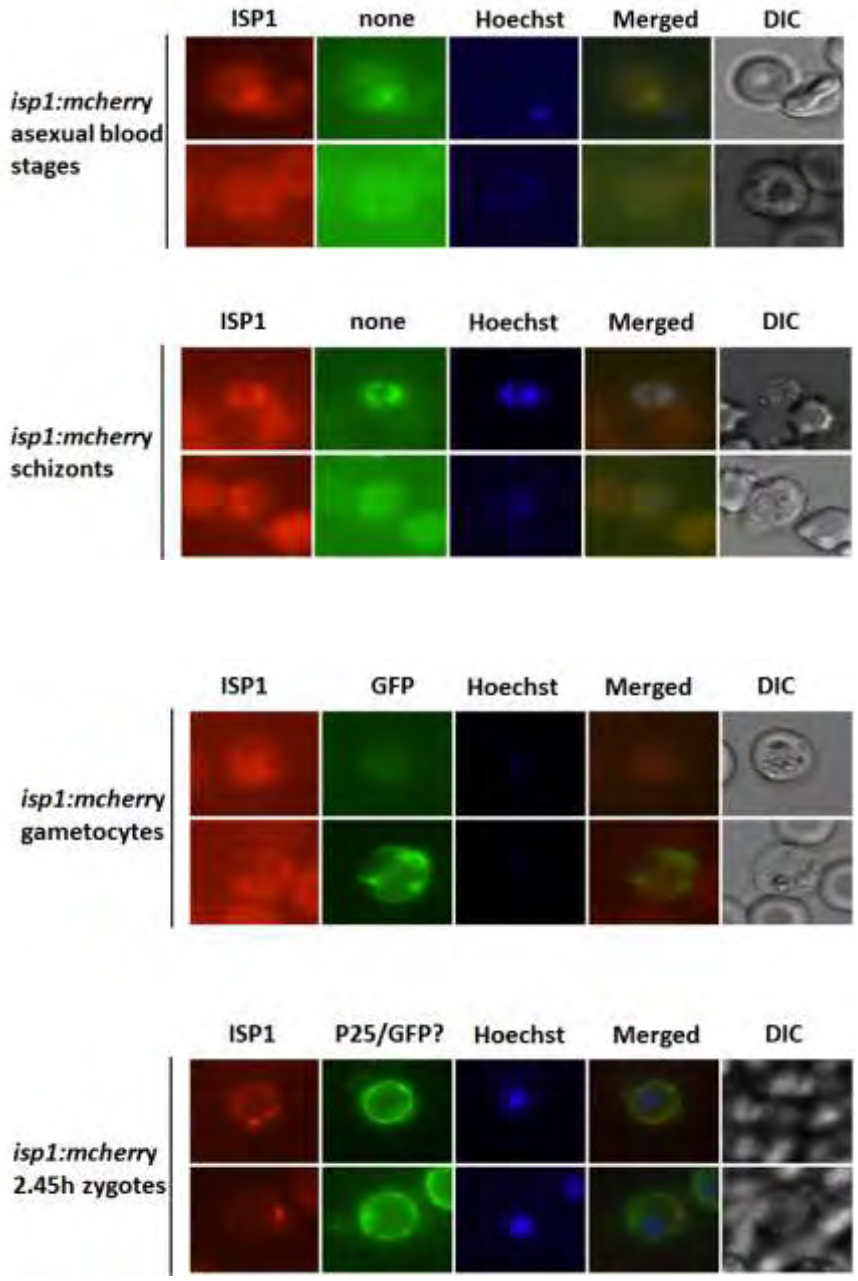


Figure K2: Expression profile of *ISP1* in mixed asexual blood stages, schizonts, gametocytes and 2.45h zygotes. *ISP1* seems to be present in gametocytes and 2.45h zygotes only, whereas no clear expression was detected in asexual stages and schizonts. Further on, *ISP1* seems to be restricted to the plasma membrane in 2.45h zygotes rather than being distributed throughout the cytoplasm.

5. Discussion

4. a) TetR-Apt system:

The Tetracycline Repressor Aptamer (TetR-apt) system is a conditional knock down system which will allow down-regulation of a functional aptamer (f-apt) tagged gene (gene of interest) in a regulatory fashion depending on the concentration of tetracycline (tet) or tetracycline analogues like anhydrotetracycline or doxycycline. TetR binds to tet or its analogue. However, in the absence of tet, TetR will bind to the hairpin loop structure formed by functional aptamer at mRNA level and therefore will interfere in the translation of the gene of interest. Advantage of this system is that it is inducible upon tetracycline addition and the gene expression can be regulated depending upon the concentration of tetracycline (J Nile group, MIT, USA - unpublished data). Upon addition of tet, TetR bound to functional aptamer hairpin loop changes shape and therefore cannot bind to the functional aptamer anymore consequently the translation will occur normally. It is assumed that in presence of high tet concentration complete restoration of translation can be achieved. In the present study it was tested whether the translational block, and therefore blocking of expression of gene of interest, is totally reversible upon addition of tet and how fast gene switch on and off is achieved. Further on, the functionality of the system in vivo (in mouse) was also checked.

For the TetR-Apt system which is under development, two independent *TetR* expressing parasite lines were generated. First parasite line which is constitutively expressing the *TetR* ($_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* line (G946)) and second parasite line which expresses the *TetR* only post activation of gams in gametocytes to ookinete stage ($_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* (G945) were generated by posing the *tetr* under the control of HSP70 promoter, p45/48 3'UTR (Figure A3) and P25 promoter and p25UTR (Figure A2), respectively. HSP70 promoter is constitutively expressed [45] while P25 promoter is expressed post activation of gametocytes till mature ookinete development [44]. Expression of the *TetR* in $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* cloned line (G946-Cl3) line was checked with Western blotting showing expression of the *TetR* in mixed asexual, schizont, gametocytes and ookinete stage (Figure G9). Further on, growth defect testing experiments were performed for his cloned line and normal growth of recombinant parasites was confirmed (Figure G8). Selectable marker was removed from $_{promoter}$*hsp70 5'UTR:2cmyc:tetr:p45/48 3'UTRs* cloned line (G946- Cl3) through negative selection (Figure G11) so that the same line can be used for further integration of *aptamer:gfp* constructs.

Expression of *TetR* in $_{promoter}$*p25 5'UTR:2cmyc:tetr:p25 3'UTRs* line (G945) was expected only after the activation of gametocytes, however, we detected *TetR* in inactivated gametocyte and 4h zygotes (Figure G2). This indicates that either some of the gametocytes have undergone pre-mature activation during preparation of samples (at room temperature 21^{o} C) or P25 UTRs are little bit leaky (showing expression at earlier stages- before activation of gametocytes as well).

Next, $_{promoter}$*pbeef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts 3'UTRs (*G967*)* (uncloned) line was generated (Figure C3), and confirmation of integration of $_{promoter}$*pbeef1aa 5'UTR:non-functional aptamer:gfp:pb dhfr/ts* plasmid into WT *HPTBB P. berghei* strain through diagnostic PCRs was achieved (Figure I1).

Attempts were made to generate $_{promoter}$*pbeef1aa 5'UTR:functional aptamer:gfp:pb dhfr/ts 3'UTRs* plasmid (see methods), however, sequencing analysis showed deletion of cytosine at position 42 (Figure B1).

Probably the mutation in the *functional aptamer* region is introduced by *E. coli* strain used for transformation. Nevertheless, bioinformatic analysis needs to be done to check if the mutation in the *functional aptamer* interferes with hairpin loop structure formation or with *TetR* binding. In case this mutation appears not to affect formation of the functional aptamer hairpin loop, this generated vector can be used for further analysis. In addition, attempts will be made to generate correct *functional aptamer* construct and parasite line simultaneously.

Also an alternative method was used in order to generate the *functional aptamer* fragment. Two oligos obtained from the 5' and two oligos obtained from the 3' region of the *functional aptamer* were annealed and subsequently combined into one fragment (Figures B2, B3). Sequencing results for the vector containing this insert remain undefined. This could be due to partial formation of the *functional aptamer* hairpin loop at DNA level.

Next, constitutive *GFP* expressing parasite line (Figure D2) was generated (uncloned) and plasmid integration PCRs were done (figure J1). This parasite line will be used for microscopic analysis to check expression of GFP and will be cloned to be used as positive control for TetR-Apt system.

Once all lines are generated, these lines will be checked for any growth defects. The *TetR* expressing lines need to be transfected with the *aptamer-gfp* (either *f-apt:gfp or nf-apt:gfp)* plasmids and will be cloned (and growth will be monitored) so that the *GFP* expression can be studied with respect to tet/ tet analogue concentration. Upon confirming the promising results, TetR-Apt system can be adapted to study gene functions in *P. berghei*.

Tetracycline has been proved to be toxic both to *T. gondii* and to *P. falciparum* [35]. However, tetracycline analogues, such as doxycycline and anhydrotetracycline are not toxic to *P. falciparum* and *T. gondii* when used at lower concentrations [35]. According to this data, toxicity of tetracycline to *P. berghei* has to be checked and if the tet proves toxic, alternative drugs such as tetracycline analogues need to be tested in *P. berghei*.

In case the system is proved to be non functional several aptamer features, such are the site of insert, thermodynamic stability, potential to form secondary structures and also sequence variations should be taken into consideration.

4.b) ISP Project

As a secondary project the study of *isp1* and *isp3* genes was chosen. ISPs (IMC sub compartment proteins) are believed to be involved in the synthesis and organization of the pellicle and the apical complex in Plasmodium species. In all Plasmodium species the formation of the IMC is essential for all morphological changes during life cycle of parasite, differentiation and invasion patterns. It has been suggested [25] that in *Plasmodium berghei ISP1* and *ISP3* are associated with the IMC indicating their role in parasites survival.

We have tried to generate two lines expressing *ISP1* and *ISP3* tagged to a fluorescence protein (*mCherry*) so that it would be easy to detect these proteins at any developmental stage. Only *isp1:mcherry* parasite line was successfully generated. Observation of this line under the fluorescence microscope showed that *ISP1* is being expressed in gametocytes and in 2.45h zygotes and expression in zygotes seems to be slightly unregulated. Further on, *ISP1* seems to be restricted to the plasma membrane in zygotes rather than being distributed throughout the whole cytoplasm.

Further observation of *ISP1* will most hopefully reveal the exact location of this protein in all developmental stages. If indeed it is proved that *ISP1* is involved in the synthesis and organization of the pellicle and restricted to the apical complex in Plasmodium species (during zygote-ookinete development) then the study of *isp1* could lead to new intervention strategies to successfully control malaria.

For *isp3:mcherry* parasite line only the transfection plasmid was prepared successfully since there was not enough time to generate the line. However, generation of this line is a future prospect.

6. Acknowledgments

I particularly want to thank Professor Andy Waters for giving me the opportunity to work in his lab for ten months and professor K. Mathiopoulos for contacting Waters Lab on my behalf. I also want to thank my supervisor Harshal Patil for guiding me throughout the whole nine months and for his patience and all members of Waters group for the daily help they provided me. Further on, I would like to especially thank Dr. Abhinar Sinha for helping me in writing this report. Last, I want to thank Erasmus Placement program for funding me for these 10 months.

7. Appendix

Name	Sequence	Descr.	R.S.	Tm (°C)
GU2668	TTGctcgagATGGAACAAAAACTCATCTCAGAAGAGGATCTGGAACAG AAGTTAATAAGTGAGGAAGACTTAgtcgacATGTCTAGATTAGATAAAA GTAAAGTGATTAAC	TetR forward primer with double myc	XhoI,SalI	54
GU2669	TATcCCgggTTAAGACCCACTTTCACATTTAAGTTGTT	TetR reverse primer	SmaI	54
GU2403	attGATATCcacgcgcgtgATCCAGGCAGAGAAAGGTCGATACGGACGGA ATGTGATGGCCTGGATAAAACACAAAACTCGAGAACATgtcgacATGG CTATGAGTAAAGGAGAAGA	GFP Forward primer with functional aptamer	EcoRV, AleI, SalI	52
GU2402	GCCcccgggTTATTTGTATAGTTCATCCATGCCAT	GFP reverse primer with stop codon	XmaI	52
GU2985	attGATATCcacgcgcgtgATCCAGGCAGAGAAAGGTC	Sense 5' oligo for functional aptamer	EcoRV	69
GU2986	CGTCCGTATCGACCTTTCTCTGCCTGGATcacgcgcgtgGATATCaat	Antisense 5' oligo for functional aptamer	EcoRV	73
GU2987	GATACGGACGGAATGTGATGGCCTGGATAAAACACAAAACTCGAGAA CATgtcgacATG	Sense 3'oligo for functional aptamer	XhoI, SalI	72
GU2988	CATgtcgacATGTTCTCGAGTTTTGTGTTTTATCCAGGCCATCACATTC	Antisense 3' oligo for functional aptamer	XhoI, SalI	69
GU2862	ATAggtaccGTTCAAGTTAAATGTCCAAAAATTATAAAAAG	sense primer to alternative 230p homology arm	KpnI	53
GU2863	GTCgaattcATATTTTCTATATTTTCGGTTGTTTGTTCATT	Antisense primer to alternative 230p homology arm	EcoRI	53
GU2404	attGATATCcacgcgcgtgATCCAGGCAGTGTAAGGTCGATACGGACGGA ATGTGATGGCCTGGATAAAACACAAAACTCGAGAACATgtcgacATGG CTATGAGTAAAGGAGAAGA	GFP Forward primer with nonfunctional aptamer	EcoRV, AleI, SalI	52
GU2923	TCAgatatcATCATGATGAGTAAAGGAGAAGAACTTTTCAC	forward primer for GFP	EcoRV	53
GU2864	TAGgcggccgcATGGGGAATATTGTATCCTGTTGTT	ISP1 (PBANKA_120940) ORF FW primer	NotI	53
GU2865	CCAggatccTTAATTTTTTTTATAATCTCTCATAATATAAATAAATAAGTT TTT	ISP1 (PBANKA_120940) ORF RV primer	BamHI	54
GU2866	ctattttgagtttttcttaatatatagagaataaaaaatGATatctttatgtttggtgaaatat aaatatttttatacaa	ISP1 (PBANKA_120940) internal sense primer	EcoRV	54
GU2867	ttgtataaaaatatttatatttcaccaaacataaagatATCatttttattctctatatattaa gaaaaactcaaaatag	ISP1 (PBANKA_120940) internal anti-sense primer	EcoRV	54

GU2921	ATCgcggccgcATATTATAAAATTGTAAATAATAAGAAACAAATAAAAAAGTAG	forward primer for ISP3 (PBANKA_132430)	NotI	54
GU2922	ATCggatccAGCAGTTAAGCAATGCTTTTTTATAAATTCATA	reverse primer for ISP3 (PBANKA_132430) without stop codon	BamHI	54
GU2969	GGATAGTTCAGATATCAAGCACTTTTCATTTAAA	internal sense upstream region of ISP3(PBANKA_132430) primer with EcoRV site introduced	EcoRV	53
GU2970	TTTAAATGAAAAGTGCTTGATATCTGAACTATCC	internal antisense upstream region of ISP3(PBANKA_132430) primer with EcoRV site introduced	EcoRV	53
GU1681	TAGTTAGCTTAAATTGTCCAACTGG	Primer for diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr* line	None	53
GU2097	GCCTTTCTCCTCCTGGAC	Primer for diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr* line	None	53
GU1682	CATAAACGGTTTATTTAAAGTCATTTTTGG	Primer for diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr* line	None	53
GU2495	AGGTACACGGCCTACAGAAA	Primer for diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr* line	None	53
GU0205	CTATTTATGAATCATTGAAGAGAC	Primer for diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr* line	None	53
GU2496	TTTCTGTAGGCCGTGTACCT	Primer for diagnostic PCRs for *hsp70 5'UTR:2cmyc:tetr* line	None	53
GU0013	TGCTCTAGAATGAATTTTAAATACAGTTTTATT	Sense primer for unrelated positive PCR control	None	53
GU0014	TGCTCTAGATTACATTACTATCACGTAAATAAC	Antisense primer for unrelated positive PCR control	None	53
GU1028	GTGAAGTTCAAATATGTGAAAAAACAATAAATGAATTTAGC	Primer for diagnostic PCRs for *non.fun.ap:gfp* and *gfp* line	None	53
GU1485	AATATTCATAACACACTTTTAAGC	Primer for diagnostic PCRs for *non.fun.ap:gfp* and *gfp* line	None	53

GU0617	CCaagcttATGTGTTTTATTTGGATGTG	Primer for diagnostic PCRs for *non.fun.ap:gfp* and *gfp* line	HindIII	53
GU0161	CATATGTTTTAATCATACCC	Primer for diagnostic PCRs for *non.fun.ap:gfp* and *gfp* line	None	53
GU2438	GGCTAGTATGAATAGCC	Primer for diagnostic PCRs for *non.fun.ap:gfp* and *gfp* line	None	53
GU2989	CAAATTAGAGAAGAAATTAAGTAAATAAATGTATA	primer for diagnostic PCR for *isp1:mChery* line	None	53
GU1934	tgtCATATGTTACTTGTACAGCTCGTCCATG	primer for diagnostic PCR for *isp1:mChery* line	NdeI	53
GU2990	AATATATATTAGTCAAAGAATGTTGAGATAAAG	primer for diagnostic PCR for *isp1:mChery* line	None	53
GU2994	TATTTTATTTCCACAATATTTATTTATTATTTATTGTTGA	primer for diagnostic PCR for *isp1:mChery* line	None	53
GU2992	ATTAGGTTTGTTTAAAAATGCATGAAGCTA	primer for diagnostic PCR for *isp1:mChery* line	None	53

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