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Presented by
M.Sc. Anja Martina Klemmer

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CRISPR interference for high-throughput gene function analysis in *Plasmodium knowlesi*

Referees: Prof. Dr. Michael Lanzer
Dr. Michael Zimmermann

Summary

Malaria is still a devastating disease that is caused by infection with unicellular parasites of the genus *Plasmodium*. In 2022, it was responsible for an estimated 249 million cases as well as an estimated 608 000 deaths worldwide. While the disease, especially cases of uncomplicated malaria, can be treated with therapeutic intervention, emergence of resistance against these antimalarial drugs has been a continuous challenge. Elucidating gene function in a systematic way has been integral for understanding the biology in other organisms in the genomic and post-genomic era. This approach has been impeded in *Plasmodium*, however, due to an extraordinarily high genomic AT-content and poor transfection efficiency in *Plasmodium falciparum*, the strain responsible for most global cases and fatalities. The latter has put large-scale genetic approaches out of reach until relatively recently, so that currently ca. 40% of the parasite's genes only have putative functional annotation, while ca. 30% have no functional annotation at all. Genetic studies that have been performed in *Plasmodium* parasites so far greatly increased our knowledge about the parasite's genome. However, due to the nature of gene knockout approaches, they could not provide functional information about genes that are essential during the asexual blood-stage of the parasite, which has been determined to be ca. half of the genome. In contrast, knockdown approaches have the capacity to provide a quantifiable phenotype for essential genes because gene function is not entirely abrogated.

Therefore, I sought to establish gene knockdown by CRISPR interference in the parasite strain *Plasmodium knowlesi* in this doctoral thesis so that this technology may eventually be used for high-throughput gene function analysis. This strain was chosen because it had exhibited exceptionally high transfection efficiency in previous reports and, thus, harbored the potential to target a large part, if not all, of the parasite genome. To this end, I used a parasite line with stable expression of the catalytically inactive version of Cas9, dCas9, that had previously been generated by Ann-Kathrin Mehnert during her Master thesis. I performed initial experiments to assess this cell line's transfection efficiency with a reporter construct, which remained inconclusive because only ca. half of transfected parasites that were resistant to the selection drug WR99210 expressed the reporter. Therefore, resistance against WR99210 was subsequently used as readout for successful transfection, revealing that parasite survival was very low and likely negatively impacted overall transfection efficiency, which I determined to be ca. 0.3%. For knockdown validation, I cloned and then transfected constructs for expression of sgRNAs targeting the essential VPS32 gene and the non-essential CEN4 and SAS6 genes into the dCas9-expressing parasite line and performed growth assays as well as transcript level

analysis via RT-qPCR. Although I could not detect a significant downregulation of VPS32 transcript levels in the respective knockdown parasite line, I identified a significant growth defect of this line compared to the control line. As expected, targeting the non-essential CEN4 gene did not elicit a growth defect, however, I detected a significant downregulation in transcripts in one of the knockdown lines compared to a control line. Targeting the SAS6 gene did not result in transcriptional downregulation in any of the generated knockdown lines. In a small-scale proof-of-principle screen, I used a library comprising 20 different sgRNA constructs targeting four genes in total: VPS32, CEN4, SAS6 and CEN3. Monitoring the constructs' relative abundance ratios over time in six conditions over two biological replicates revealed that I could not detect a specific and efficient knockdown effect in this screen with the CRISPR interference system that I employed.

In conclusion, I was able to determine the transfection efficiency of the *P. knowlesi* dCas9 line used in this thesis, which was lower than published efficiencies for the *P. knowlesi* wildtype strain but sufficiently high to, in theory, facilitate screens of moderately large scale. Additionally, I was able to show a significant downregulation of CEN4 transcripts in one knockdown parasite line as well as a significant growth defect in the VPS32 knockdown line. Unfortunately, the results of two replicates of a small-scale proof-of-principle screen revealed that I was not able to detect specific and efficient knockdown via the CRISPR interference system used in this thesis. Further inquiry into how efficient CRISPR interference works in this parasite strain and potentially subsequent development of technology is thus necessary to validate it as a tool for high-throughput genetic screening approaches.

Zusammenfassung

Noch immer ist Malaria eine verheerende Krankheit, die durch die Infektion von einzelligen Parasiten der Gattung *Plasmodium* ausgelöst wird. Allein im Jahr 2022 war sie verantwortlich für geschätzt 249 Millionen Krankheits- und 608 000 Todesfälle weltweit. Obwohl die Krankheit, insbesondere in unkomplizierten Fällen, medikamentös behandelt werden kann, stellt das Auftreten von Resistenzen gegenüber dieser Malariamittel eine kontinuierliche Herausforderung dar. Um die Biologie anderer Organismen zu verstehen, war das systematische Entschlüsseln von Genfunktion wesentlich in den Zeitaltern der Genomik und Postgenomik. Dieser Ansatz wurde jedoch durch einen außergewöhnlich hohen AT-Gehalt des Genoms und eine sehr geringe Transfektionseffizienz des Parasitenstamms *Plasmodium falciparum*, der für die weltweit meisten Krankheits- und Todesfälle verantwortlich ist, stark eingeschränkt. Die geringe Transfektionseffizienz hat bis vor kurzer Zeit genetische Ansätze in größerem Ausmaß außer Reichweite gehalten, sodass aktuell ca. 40% der Gene des Parasiten lediglich eine mutmaßliche funktionelle Annotation haben und ca. 30% keinerlei funktionelle Annotation erhalten haben. Genetische Studien, die bisher mit dem *Plasmodium*-Parasiten durchgeführt wurden, konnten unser Wissen über dessen Genom deutlich erweitern. Aufgrund der Natur von Gen-Knockout-Ansätzen konnten sie jedoch keine funktionellen Informationen über Gene liefern, die während des asexuellen Blutstadiums des Parasiten essenziell sind, welche etwa die Hälfte des Parasiten-Genoms ausmachen. Im Gegensatz dazu können Knockdown-Ansätze einen quantifizierbaren Phänotyp für essenzielle Gene liefern, da die Genfunktion nicht vollständig aufgehoben wird.

Aus diesem Grund habe ich in dieser Doktorarbeit den Versuch unternommen, Gen-Knockdown durch CRISPR-Interferenz im Parasitenstamm *Plasmodium knowlesi* zu etablieren, damit diese Technologie schließlich für die Hochdurchsatz-Gen-Funktionsanalyse verwendet werden kann. Dieser Stamm wurde ausgewählt, weil er in früheren Publikationen eine außergewöhnlich hohe Transfektionseffizienz gezeigt hatte. Daher bot er das Potenzial, einen Großteil des Genoms oder möglicherweise das gesamte Genom abdecken zu können. Zu diesem Zweck habe ich eine Parasitenlinie mit stabiler Expression der katalytisch inaktiven Version von Cas9, dCas9, verwendet, die zuvor von Ann-Kathrin Mehnert im Rahmen ihrer Masterarbeit generiert wurde. Ich führte erste Experimente durch, um die Transfektionseffizienz dieser Zelllinie mit einem Reporterkonstrukt zu erfassen. Diese ließen jedoch keine eindeutigen Aussagen zu, da nur etwa die Hälfte der transfizierten Parasiten den Reporter exprimierten, obwohl sie resistent gegen die Selektionsdroge WR99210 waren.

Aufgrund dessen habe ich anschließend die Resistenz gegen WR99210 als Indikator für eine erfolgreiche Transfektion benutzt. Dabei zeigte sich, dass die Überlebensrate der Parasiten äußerst gering war und sich wahrscheinlich negativ auf die gesamte Transfektionseffizienz auswirkte, die meinen Berechnungen zufolge 0.3% betrug. Zur Knockdown-Validierung transfizierte ich Konstrukte zur Expression von sgRNAs, die auf das essenzielle VPS32-Gen und die nicht-essenziellen CEN4- und SAS6-Gene abzielten, in die dCas9-exprimierende Parasitenlinie und führte Wachstumsanalysen sowie eine Analyse der Transkriptlevel mittels RT-qPCR durch. Obwohl ich in der VPS32-Knockdown Parasiten-Zelllinie keine signifikante Herabregulierung des VPS32-Transkriptlevel feststellen konnte, stellte ich im Vergleich zur Kontrolllinie einen signifikanten Wachstumsdefekt dieser Linie fest. Wie erwartet löste das Targeting des nicht-essenziellen CEN4-Gens keinen Wachstumsdefekt aus. Dafür konnte ich im Vergleich zu einer Kontroll-Linie eine signifikante Herabregulierung der Transkripte in einer der CEN4-Knockdown-Linien feststellen. Das Targeting des SAS6-Gens führte in keiner der erzeugten Knockdown-Linien zu einer Herabregulierung der Transkription. In einem kleinen Screen habe ich eine 20 verschiedene sgRNA-Konstrukte umfassende Plasmid-Bibliothek transfiziert, die auf insgesamt vier Gene abzielten: VPS32, CEN4, SAS6 und CEN3. Das Analysieren der relativen Mengenverhältnisse dieser Konstrukte in sechs Bedingungen über zwei biologische Replikate und der verschiedenen Zeitpunkte hinweg ergab, dass ich in diesem Screen mit dem von mir verwendeten CRISPR-Interferenzsystem keinen spezifischen und effizienten Knockdown-Effekt feststellen konnte.

Zusammenfassend konnte ich die Transfektionseffizienz der in dieser Arbeit verwendeten *P. knowlesi*-dCas9-Linie bestimmen, die niedriger war als die veröffentlichten Effizienzen für den *P. knowlesi*-Wildtyp-Stamm, jedoch ausreichend hoch, um Screens in mittelgroßem Maßstab zu ermöglichen. Darüber hinaus konnte ich eine signifikante Herabregulierung der CEN4-Transkripte in einer Knockdown-Parasitenlinie sowie einen signifikanten Wachstumsdefekt in der VPS32-Knockdown-Linie nachweisen. Leider zeigten die Ergebnisse von zwei Replikaten des Proof-of-Principle-Screens, dass ich mit dem in dieser Arbeit verwendeten CRISPR-Interferenzsystem kein spezifisches und effizientes Knockdown feststellen konnte. Daher ist es notwendig weiterhin zu untersuchen, wie effiziente CRISPR-Interferenz in diesem Parasitenstamm funktioniert und gegebenenfalls fehlende Technologien zu entwickeln, damit man es als Tool für genetische Screens im Hochdurchsatz-Verfahren validieren kann.

List of Abbreviations

A (nucleobase)	Adenine
BF	Brightfield
bp	Base pair
BroI	BRO1 domain-containing protein
BSA	Bovine serum albumin
C (nucleobase)	Cytosine
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
Cas	CRISPR-associated genes
cDNA	Complementary DNA
CDS	Coding sequence
Cen (protein)	Centrin
CEN (gene)	Centrin
ChIP-qPCR	Chromatin immunoprecipitation quantitative PCR
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
cRPMI	Complete RPMI
crRNA	CRISPR RNA
CRT	Chloroquine resistance transporter
Ct	Threshold cycle
DARC	Duffy antigen receptor for chemokines
DBP	Duffy-binding protein
dCas9	dead Cas9 (catalytically inactive)
ddH₂O	Deionized distilled water
DHFR	Dihydrofolate reductase
DIC	Differential interference contrast
DiCre	Dimerizable Cre recombinase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOZI	Development of zygote inhibited
DPBS	Dulbecco's Phosphate Buffered Saline
DSB	Double strand break
EBA-175	Erythrocyte binding antigen-175
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced GFP
ESCRT	Endosomal sorting complex required for transport
EuPaGDT	Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool
F (primer)	Forward primer
FACS	Fluorescence activated cell sorting

FBPA	Fructose-bisphosphate aldolase
FCS	Forward scatter
Fiji	Fiji is just ImageJ
FKBP	FK506-binding protein
FPKM	Fragments per kilobase million
FRB	FKBP-rapamycin binding
Fy	Duffy antigen
G (nucleobase)	Guanine
gDNA	Genomic DNA
GFP	Green fluorescent protein
glmS	Glucosamine-6-phosphate activated ribozyme
gRNA	Guide RNA
HA	Hemagglutinin
hDHFR	Human DHFR
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNH	Catalytic domain in Cas9
hpi	Hours post invasion
IC₅₀	Half maximal inhibitory concentration
IDC	Intraerythrocytic developmental cycle
IFA	Immunofluorescence assay
iRBC	Infected RBC
iRPMI	Incomplete RPMI
kb	Kilobase
kDa	Kilodalton
KRAB	Krüppel associated box
LB	Lysogeny broth
loxP	Locus of X-over P1
<i>M. fascicularis</i>	<i>Macaca fascicularis</i>
<i>M. mulatta</i>	<i>Macaca mulatta</i>
<i>M. nemestrina</i>	<i>Macaca nemestrina</i>
MACS	Magnetic activated cell separation
MFS	Mutagenesis fitness score
MIS	Mutagenesis index score
MTOC	Microtubule organizing center
NaBH₄	Sodium borohydride
NaCl	Sodium chloride
NF54	Nijmegen <i>falciparum</i> strain 54
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
ORF	Open reading frame
<i>P. berghei</i>	<i>Plasmodium berghei</i>
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
<i>P. knowlesi</i>	<i>Plasmodium knowlesi</i>

<i>P. malariae</i>	<i>Plasmodium malariae</i>
<i>P. ovale</i>	<i>Plasmodium ovale</i>
<i>P. vivax</i>	<i>Plasmodium vivax</i>
<i>P. yoelii</i>	<i>Plasmodium yoelii</i>
PAM	Protospacer adjacent motif
<i>Pb</i>	<i>P. berghei</i>
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
<i>Pf</i>	<i>P. falciparum</i>
PFA	Paraformaldehyde
PlasmoGEM	<i>Plasmodium</i> genetic modification
poly(A)	Sequence of multiple adenosine monophosphates
pre-crRNA	Pre-CRISPR RNA
<i>Py</i>	<i>P. yoelii</i>
<i>pyalba4::gfp</i>	ALBA4 gene tagged with GFP in <i>P. yoelii</i>
R (primer)	Reverse primer
RBC	Red blood cell
RGR	Ribozyme-guide-ribozyme
RH4	Reticulocyte binding protein homologue 4
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
RPMI	Roswell Park Memorial Institute [cell culture medium]
rRNA	Ribosomal RNA
RuvC	Catalytic domain in Cas9
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pyogenes</i>	<i>Streptococcus pyogenes</i>
Sas6 (protein)	Spindle abnormal protein 6
SAS6 (gene)	Spindle abnormal protein 6
SD	Standard deviation
sgRNA	Single guide RNA
SOC	Super optimal medium with catabolic repressor
spp.	species
SSC	Side scatter
T (nucleobase)	Thymine
<i>T. brucei</i>	<i>Trypanosoma brucei</i>
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
TE	TRIS-EDTA
TetR	Tet repressor
<i>Tg</i>	<i>T. gondii</i>
TPM	Transcript per million
tracrRNA	trans-acting crRNA
TRIS	Tris(hydroxymethyl)aminomethane
TSS	Transcription start site

UTR	Untranslated region
VP64	Protein domain used for CRISPRa
Vps32 (protein)	Vacuolar protein sorting associated protein 32
VPS32 (gene)	Vacuolar protein sorting associated protein 32
w/v	Weight per volume
WHO	World Health Organization
WR	WR99210
WT	Wildtype
yFCU (gene)	Yeast cytosine deaminase and uridyl-phosphoribosyltransferase

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1. Introduction

1.1 Malaria

Malaria is an infectious disease, caused by a unicellular protozoan parasite of the genus *Plasmodium* (Sato, 2021), that has afflicted humankind for a very long time. The origin of the name that is still in use today is believed to be “mal’aria”, old Italian for “bad air”, reminiscent of a time when people were convinced that the cause of the disease was miasmas surrounding swamps and marshes (Hempelmann and Krafts, 2013). There are even references in ancient texts from long before that time that some historians suppose describe malaria. These originated from different countries and parts of the world, including China (dating back to 2700 BC) and Egypt, as well as later Greece, Rome and the Arab Empire (Cox, 2002; Hempelmann and Krafts, 2013). The belief that malaria was contracted through toxic miasmas was only seriously refuted by two important discoveries made in the late 19th century. Firstly, the French army doctor Charles Louis Alphonse Laveran discovered *Plasmodium* parasites in infected blood using a microscope in 1880 and, secondly, in the following ten to twenty years, Ronald Ross and several Italian scientists, including Giovanni Battista Grassi, separately identified mosquitoes to be the vectors transmitting these parasites (Cox, 2002; Cox *et al.*, 2010).

Although the disease has been a part of human history maybe from its very beginning it is unfortunately still far from eradicated today. According to the latest World malaria report by the World Health Organization (WHO) (2023) it is the most prevalent infectious disease in the world with an estimated ca. 50% of the world’s population being at risk of contracting it (living in areas where transmission occurs). There have been an estimated 249 million cases of malaria and an estimated 608,000 deaths in 2022 (World Health Organization, 2023). While these cases have been recorded in 85 malaria-endemic countries and regions around the world, the distribution of cases is very unequal (Fig.1.1). The vast majority (94%) of all global cases in 2022 occurred in the WHO African Region and only four countries recorded almost 50% of all cases: Nigeria (27%), the Democratic Republic of the Congo (12%), Uganda (5%) and Mozambique (4%) (World Health Organization, 2023).

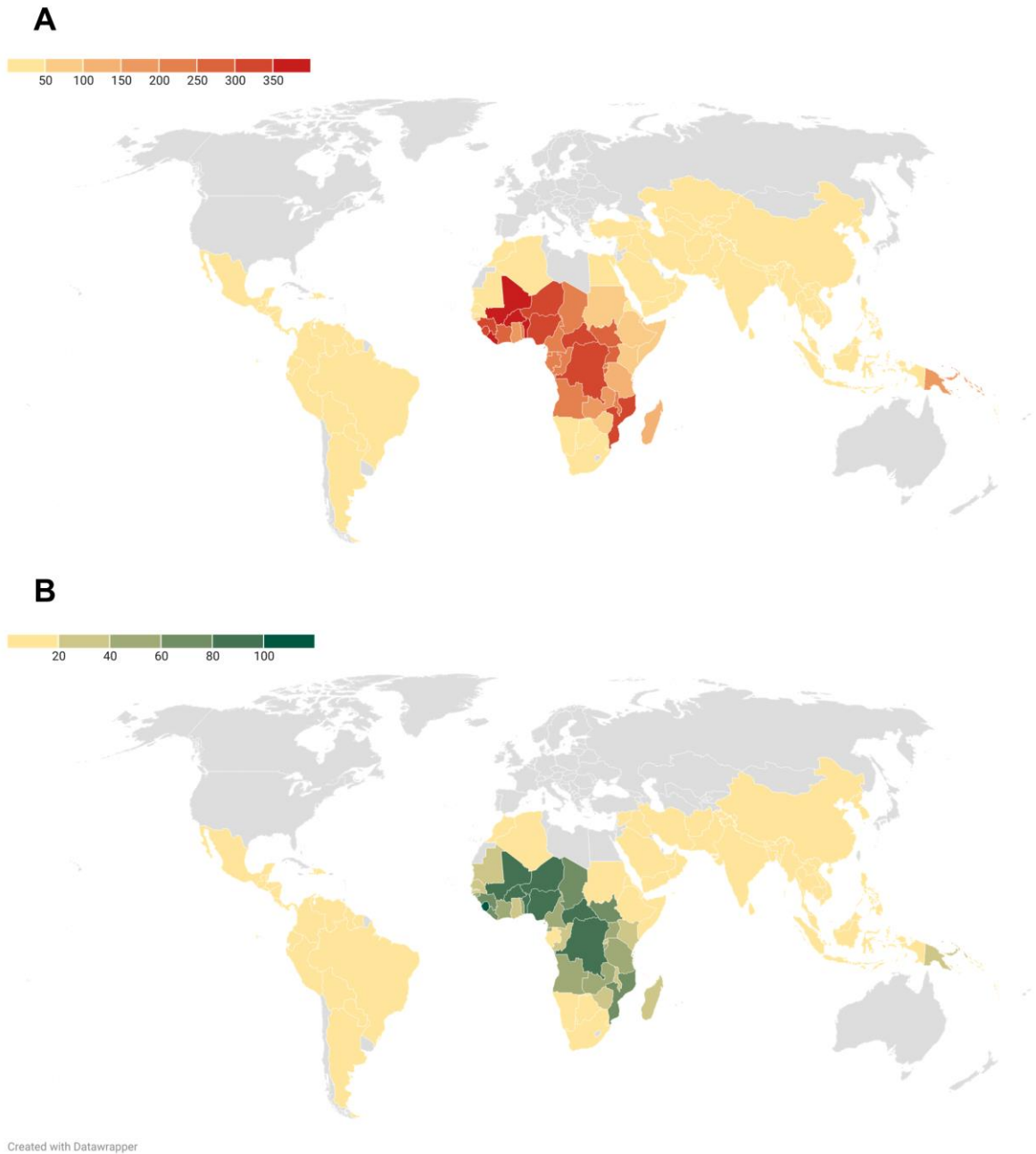


Fig. 1.1: Estimated malaria incidence and mortality rate in 2020. (A) and (B) The color each country is depicted in represents the estimated malaria incidence per 1000 population at risk (A) or estimated malaria mortality rate per 100,000 population (B) according to the respective color-scale. For countries in grey data is not applicable. This figure was created with Datawrapper using data from the Global Health Observatory (WHO website).

Of more than 200 *Plasmodium* species (spp.) that have been identified, five are known to be able to infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and the zoonotic *Plasmodium knowlesi*. The strain that is the most prevalent, especially in sub-Saharan Africa, and that is responsible for almost all global deaths is *P. falciparum* (Varo, Chaccour and Bassat, 2020). The second most prevalent strain is *P. vivax* which is found in many areas and regions of the world but is especially prevalent in Asia as well as South and Central

America (Poespoprodjo *et al.*, 2023). Infections with the remaining three strains are rather rare (Poespoprodjo *et al.*, 2023). However, infections with *P. knowlesi* are on the rise in Southeast Asia, especially in Malaysia where it is currently the only non-imported cause of malaria, and due to the zoonotic nature of infection threatens the malaria elimination efforts in that area (Chin *et al.*, 2020).

Infections with *Plasmodium* parasites begin with a female *Anopheles* mosquito taking a blood meal and thereby releasing parasites into the skin of the affected individual (for a more detailed description of the parasite's lifecycle, see chapter 1.2). The symptomatic stage then starts ca. 7-14 days later, when the parasites have reached the bloodstream (White *et al.*, 2014; Phillips *et al.*, 2017). In the beginning of the infection, the symptoms are often not specific to malaria, including a general feeling of malady, muscle aches and low-grade fever (White *et al.*, 2014; Varo, Chaccour and Bassat, 2020).

The symptoms that are most characteristic for malaria are fever paroxysms that occur every 48 h for *P. falciparum*, *P. vivax* and *P. ovale*, every 72 h for *P. malariae* and every 24 h for *P. knowlesi* (Coatney *et al.*, 1971; Varo, Chaccour and Bassat, 2020). The fevers are caused by massive parallel hemolysis (Phillips *et al.*, 2017) which occurs when newly formed daughter cells of the parasite burst out of red blood cells (RBCs), at the end of the parasite's asexual replication cycle in the blood (Mideo *et al.*, 2013; Rijo-Ferreira *et al.*, 2020) (see 1.2). This releases malaria endotoxin which is a complex of the malaria pigment (called hemozoin) and parasite DNA which, in turn, triggers an immune response that ultimately manifests as fever (Phillips *et al.*, 2017). When patients could not have developed a high level of immune protection (yet), as is the case in children under 5 years of age or individuals of all ages in low-transmission settings, severe forms of the disease can occur (White *et al.*, 2014; Phillips *et al.*, 2017; Poespoprodjo *et al.*, 2023). These include severe malarial anemia, which is the most common manifestation of severe malaria or, especially in *P. falciparum*-caused infections, cerebral malaria (Moxon *et al.*, 2019; Poespoprodjo *et al.*, 2023). The latter is characterized by sequestration of infected RBCs in brain capillaries and the cause of death for cerebral malaria has been indicated to be brain swelling which leads to deterioration of brain stem function (Moxon *et al.*, 2019). Malaria, especially exhibiting uncomplicated clinical symptoms, can be treated with appropriate therapeutic intervention. However, resistance to antimalarial compounds has been an ongoing challenge, so that, e.g., resistance to the drug chloroquine in *P. falciparum* is present in almost all its transmission areas except in Central America and is continuously developing in *P. vivax* (Poespoprodjo *et al.*, 2023). Recently, the *P. falciparum* strain has also exhibited partial resistance

to artemisinin, which is part of the current frontline antimalarial therapeutic intervention artemisinin combination therapy (Poespoprodjo *et al.*, 2023).

As implied earlier, transmission settings can be described as high/perennial (stable) or low/seasonal (unstable). This is dependent on if transmission occurs during the whole year or if it is restricted to several months (usually during rainy season) and individuals are infected generally less (Phillips *et al.*, 2017). That means, if children living in a stable transmission area survive the first few years of their life, they gradually build up a partial immunity towards the infection which eventually becomes asymptomatic in adults (Phillips *et al.*, 2017). This is the reason why there is still hope for a high efficacy protective malaria vaccine. So far, two vaccines have been recommended by the WHO for widespread use: RTS,S in 2021 and recently R21 in 2023 (World Health Organization, 2023). RTS,S has shown only moderate efficacy in phase III clinical trials, where 3 doses and a booster were administered to young children and reduced infections over 3-4 years by 36% or 26%, depending on how young the children were when the regimen was started (RTS, 2015; Poespoprodjo *et al.*, 2023). However, in combination with chemoprophylactic measures, RTS,S has shown notable decreases in malaria morbidity and mortality (Chandramohan *et al.*, 2021; Poespoprodjo *et al.*, 2023). R21 has demonstrated relatively high efficacy with a 12-months vaccine efficacy of 75% at seasonal sites and 68% at perennial sites (Dattoo *et al.*, 2024). Another advantage is that it is reportedly possible to manufacture R21 at lower cost and higher scale compared to the RTS,S vaccine (Lambach *et al.*, 2024). Another means of disease prevention that has proved very successful in the past is vector control. The most common measures are insecticide-treated bed nets and indoor residual spraying which has prevented malaria in several areas (Phillips *et al.*, 2017). However, adaptation in the vector has caused resistance to insecticides, so that of 78 countries that monitor resistance to insecticides in mosquitoes, 60 have reported the prevalence of resistance to one or more insecticides since 2010 (Phillips *et al.*, 2017).

In the first ca. 20 years of this millennium, the efforts towards malaria eradication have made notable progress. For example, the global case incidence decreased from 81.0 per 1000 population at risk in 2000 to 56.8 per 1000 population in 2019. Importantly, the mortality rate decreased by ca. 50% in this timeframe (World Health Organization, 2023). The WHO accredits this development to mainly insecticide-treated bed nets and artemisinin combination therapy, but also indoor residual spraying of insecticides (World Health Organization, 2016; Phillips *et al.*, 2017). Unfortunately, this progress has stagnated in the last few years, which the WHO attributes to complications and disruptions in healthcare during the COVID19 pandemic from 2020-2022 (World Health Organization, 2023). Another worrying development is that in 2022

global funding for basic malaria research and development decreased to its lowest recorded level since 2007 (World Health Organization, 2023). However, developing highly effective drugs targeting the parasite as well as identifying high efficacy vaccine candidates requires a thorough understanding of the fundamental biology of *Plasmodium* biology. With resistances to antimalarial drugs and insecticides on the rise, it can be argued that it is now more important than ever to enable the research necessary for that endeavor.

1.2 The life cycle of *Plasmodium* parasites

Plasmodium parasites have a complex life cycle in which they alternate between a mosquito vector and a vertebrate host (Fig. 1.2 A). Asexual replication in the latter can lead to rapidly increasing numbers of infected RBCs, the percentage of which is called parasitemia. Understanding the parasite's proliferation strategies during all stages of its life cycle is crucial. However, it is during the asexual blood-stage that the parasite establishes acute infection in the human host through massive proliferation which is the underlying precondition to all morbidity and mortality associated with malaria (Voß et al., 2023). Therefore, this stage of the life cycle harbors a unique potential to find targets for therapeutic intervention or vaccine candidates.

In humans, when a *Plasmodium*-infected female *Anopheles* mosquito takes a blood meal from an individual, several sporozoites, which represent a highly motile form of the parasite, are injected into the skin (dermis) (Cowman *et al.*, 2016; Frischknecht and Matuschewski, 2017; Venugopal *et al.*, 2020; Sato, 2021) (Fig. 1.2 A (a)). From there, sporozoites can reach a blood vessel, travel in the bloodstream to the liver and infect hepatocytes (Frischknecht and Matuschewski, 2017; Venugopal *et al.*, 2020) (Fig. 1.2 A (b)). There, the parasite undergoes a massive reproductive expansion so that within a single hepatocyte, tens of thousands new daughter cells (called merozoites) can be produced (Sato, 2021). Upon bursting of the hepatocyte, the merozoites are released back into the bloodstream and start invading RBCs (Venugopal *et al.*, 2020; Sato, 2021) (Fig. 1.2 A (c)).

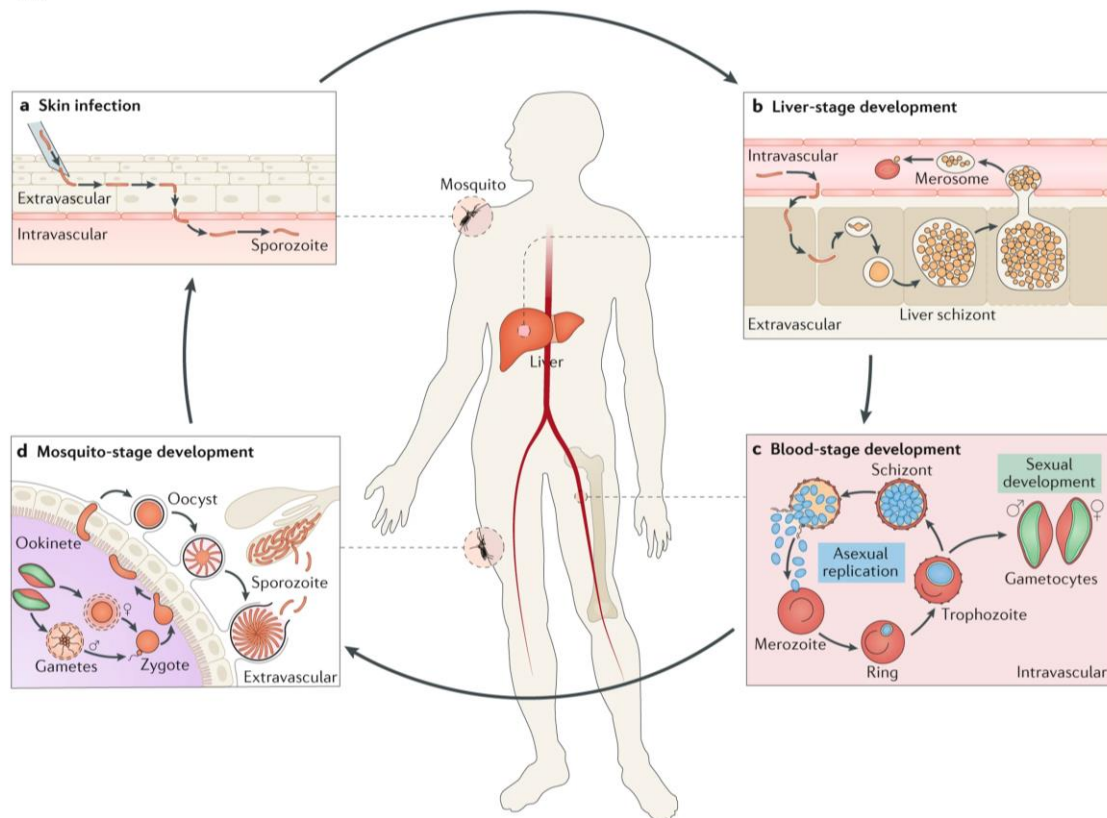
During invasion of the RBC, the parasite begins to form a parasitophorous vacuole membrane that will later engulf it (Lingelbach and Joiner, 1998). Over the course of the intraerythrocytic development cycle (IDC), which itself can be divided into different stages representing the parasite's development (Fig. 1.2 B), the parasite continuously rearranges the cytoskeletal architecture of the host cell. It also progressively claims space and resources from the RBC to accommodate its needs concerning growth, replication and host immune evasion (Maier *et al.*, 2009). Initially, the parasite is visible as a ring in thin blood smears (stained with e.g. Giemsa)

which is why this stage is called the ring-stage (Fig. 1.2 B). The second stage is called the trophozoite stage (Fig. 1.2 B), derived from the Greek word *trophía* for nourishment. Here, the parasite grows in size by metabolizing nutrients from the host cell's cytoplasm, and through specialized pathways from the environment beyond the RBC (Desai, Bezrukov and Zimmerberg, 2000; Desai, 2023). During this process, the parasite metabolizes most of the RBC's hemoglobin, which it uses as a source of amino acids (Goldberg, 2013). Since the thereby produced free heme is toxic to the parasite, it detoxifies and stores the heme in an inert crystalline form called hemozoin (Goldberg, 2013). In preparation for producing new merozoites, the parasite also starts replicating its genome. The prevailing perspective on the progression of the IDC has been that the schizont-stage begins when there are three separate nuclei visible (Fig. 1.2 B). Recently, a new perspective was offered in which the schizont-stage begins with the onset of genome replication (Voß et al., 2023). During this process, which is called schizogony, multiple rounds of DNA replication and asynchronous nuclear division occur, followed by a last synchronous one, without cytokinesis, leading to a multinucleated cell (Gerald, Mahajan and Kumar, 2011; Francia and Striepen, 2014; Simon, Stürmer and Guizetti, 2021). Towards the end of the IDC, the size of the parasite fills almost the entire RBC. During the last instances of schizogony the schizont undergoes segmentation (Fig. 1.2 B) involving cytokinesis to form up to 30 new merozoites – the exact number varies and depends on a multitude of factors, likely including resources available to the parasite (Stürmer *et al.*, 2023). The process in which the merozoites then exit the RBC is called egress (Fig. 1.2 B). Through the concerted effort of many signaling and effector proteins, including kinases such as protein kinase G, and perforin-like proteins, as well as proteases, the parasite manages to escape first the parasitophorous vacuole and then the RBC (Dvorin and Goldberg, 2022). As introduced in chapter 1.1, the fever paroxysms characteristic to the disease are caused by this process of parasite egress from RBCs, which happens synchronously in the blood of the human host, and the subsequent reaction of the host's immune system. In most human-infectious *Plasmodium* strains, the asexual blood-stage cycle lasts 48 h (*P. falciparum*, *P. vivax*, *P. ovale*), 72 h in *P. malariae* and 24 h in *P. knowlesi*.

A small fraction of all parasites present in the bloodstream undergo sexual differentiation and become gametocytes (Venugopal *et al.*, 2020) (Fig. 1.2 A (c)). If these gametocytes are taken up by a mosquito during a blood meal, female and male gametocytes produce a macrogamete and eight microgametes, respectively, that can then fuse and become a zygote in the midgut of the mosquito (Venugopal *et al.*, 2020) (Fig. 1.2 A (d)). The zygote then transforms into a motile form called ookinete that traverses the midgut epithelium and settles as an oocyst inside which

thousands of sporozoites develop (Frischknecht and Matuschewski, 2017; Venugopal *et al.*, 2020). When these are released into the circulatory fluid of the mosquito (hemolymph) and thereby reach and enter the salivary glands, the cycle can start anew when the mosquito takes another blood meal (Frischknecht and Matuschewski, 2017; Venugopal *et al.*, 2020).

A



B

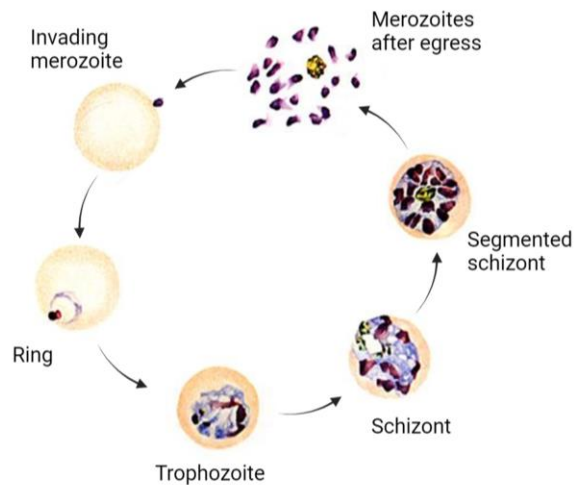


Fig. 1.2: The *Plasmodium* life cycle. (A) Through the bite of an infected female *Anopheles* mosquito sporozoites first reach the dermis and then the bloodstream of an individual (a). Sporozoites reach the liver and form a liver schizont that contains tens of thousands of merozoites (b). Merozoites then escape merosomes into the bloodstream where they invade RBCs which starts the asexual blood-stage development (c). Inside the RBC, the parasite grows and undergoes schizogony, thereby producing up to 30 new merozoites that egress from the RBC and that can start the cycle anew. Some parasites undergo sexual development into female and male gametocytes, respectively, that also cycle in the blood. A mosquito can take up these gametocytes with its blood meal. In the midgut of the mosquito the gametocytes develop into gametes and fuse to form a zygote that, in turn, develops

into a mobile ookinete which eventually settles into an oocyst (d). Inside the oocyst, new sporozoites are produced which, after egress, reach the salivary glands of the mosquito. From there the transmission cycle can start anew. This panel was taken from (Venugopal *et al.*, 2020) (B) Stages of asexual blood-stage cycle of *P. falciparum* (painted) as seen on a thin blood smear through a microscope after staining with e.g. Giemsa stain. After the parasite has invaded the RBC it is visible as a ring. Subsequently, the parasite grows in size during the trophozoite stage. Upon replication of its genome (here the nuclei are depicted as dark red spots in blue-purple cytoplasm), the parasite is referred to as a schizont. The malaria pigment (or hemozoin) is shown in yellow and black. At the end of replication, the schizont segments and nuclei are packaged into new merozoites that then egress from the RBC. Free merozoites invading RBCs constitutes the beginning of a new cycle. This panel was created with BioRender.com using modified images from (Coatney *et al.*, 1971).

1.3 *Plasmodium knowlesi*

1.3.1 Zoonosis and adaptation to cell culture

The first extensive description of *P. knowlesi* parasites was made by Robert Knowles, after whom the strain was later named, together with Das Gupta (Knowles and Das Gupta, 1932). In their experiments, they infected a variety of different primate species and found varying susceptibilities to *P. knowlesi* in them. Knowles and Das Gupta also recognized that while, generally, the parasites' morphology resembled *P. vivax* or *P. malariae*, the early blood stages (ring- and trophozoite-stages) could also be identified as *P. falciparum*. It should be noted that as part of this study the parasite was also transferred to humans by infecting three human volunteers*. While the course of infection was varied among this small group of subjects (two showed mild courses of disease and one showed severe symptoms), all three recovered spontaneously without the administration of antimalarial drugs (Knowles and Das Gupta, 1932).

In its natural hosts, the long-tailed and pig-tailed macaques (*Macaca fascicularis* and *Macaca nemestrina*, respectively), *P. knowlesi* causes mild infections in contrast to severe infections in its artificial host, rhesus monkeys (*Macaca mulatta*), with the vast majority leading to death (Coatney *et al.*, 1971; Singh and Daneshvar, 2013). Before molecular methods, such as nested polymerase chain reaction (PCR), were available, injection of *Plasmodium*-infected blood into rhesus monkeys and subsequent observation of the course of infection was the only accessible method to relatively reliably identify a *P. knowlesi* infection (Lee *et al.*, 2009). In fact, this procedure was partly how Chin *et al.* deduced that the first recorded naturally acquired infection with *P. knowlesi* in a human was indeed with *P. knowlesi* in 1965. In the corresponding publication, it was described that parasites had first been found in a blood smear of a man returning to the United States after having spent four weeks in Malaysia (Chin *et al.*, 1965). Based on the parasites' morphology, as inspected by microscopy, the initial diagnosis of *P. falciparum* was changed to

* Historical context: the term "volunteer" was adopted from the original text. Since there is no further information regarding ethical circumstances of these experiments available in the paper, it is not possible to assess if this term would be appropriate for today's standard of ethical scientific conduct.

P. malariae instead. Nonetheless, in further experiments with infected blood samples of this patient, human volunteers[†] were infected and, in turn, from their infected blood several species of monkeys, including rhesus monkeys, were successfully infected. In addition, the researchers found fever rhythms of the quotidian type (peaks appearing every 24 h) which is distinct from the tertian type (intervals of 48 h) typical of *P. falciparum*, *P. ovale*, and *P. vivax* and also distinct from the quartan type (intervals of 72 h), occurring in *P. malariae* infections (Coatney *et al.*, 1971). The cumulative evidence of the parasites' morphology, the pattern of fever (peaks) in the host and the fact that they caused strong infections in rhesus monkeys led to their conclusion that the initial infection had been a zoonosis with *P. knowlesi* parasites (Chin *et al.*, 1965).

As of today, evidence strongly suggests that infections with *P. knowlesi* remain zoonotic and that at least no sustained human-vector-human transmission occurs (Fornace *et al.*, 2023; Lee *et al.*, 2022; Ruiz Cuenca *et al.*, 2022; B. Singh & Daneshvar, 2013). Nonetheless, human infections with *P. knowlesi* have become reason for concern over the last years (Varo, Chaccour and Bassat, 2020; World Health Organization, 2023). While there are great public health efforts made in Malaysia (Chin *et al.*, 2020), which is the country that counts the highest number of *P. knowlesi* malaria cases (Lee *et al.*, 2022), the rate of severe/life-threatening cases has been reported in one systematic study to have reached that of *P. falciparum* malaria (Daneshvar *et al.*, 2009; Grüning *et al.*, 2014).

Fortunately, the *P. knowlesi* malaria case numbers remain much lower than *P. falciparum* or *P. vivax*. An increase in cases has been linked to human activity, namely progressively advancing into areas that are the habitat of both the parasite's natural hosts and *P. knowlesi*-transmitting vectors (Singh & Daneshvar, 2013) (Fig. 1.3). Therefore, while *P. knowlesi* malaria cases contribute only a small part to all malaria cases worldwide, as the only human-infecting zoonotic malaria parasite, it occupies a special position in the worldwide initiative to eradicate malaria (Chin *et al.*, 2020; Lee *et al.*, 2022; WHO, 2023).

[†] Historical context: volunteers were inmates. Regarding ethical scientific conduct, the same applies as in footnote above.

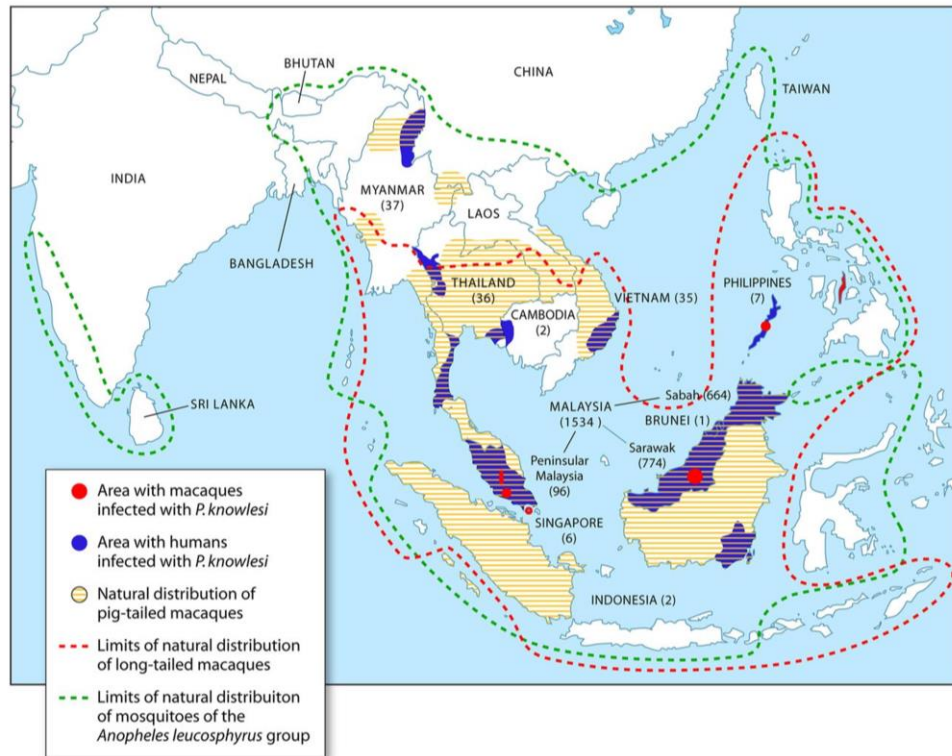


Fig. 1.3: Human *P. knowlesi* infections occur in the bounds of the natural distribution of long- and pig-tailed macaques, as well as of the *P. knowlesi*-transmitting *Anopheles leucosphyrus* group. Shown here are areas where cases of human (blue areas) or macaque (red circles) *P. knowlesi* infections have been recorded in Southeast Asia in 2013. Human case numbers are given in brackets per country. For most human cases, the natural distribution of the *P. knowlesi*-transmitting *Anopheles* group (area within the green dotted line) and the distribution of its natural host, pig-tailed macaques (yellow shaded area) overlap. Therefore, Singh and Daneshvar (2013) hypothesized that humans progressively advancing into the natural habitat/distribution area of these macaques and mosquitoes, respectively, harbors the risk of increased human *P. knowlesi* infections. The figure was taken from (Singh and Daneshvar, 2013).

Early studies utilizing animal models infected with *P. knowlesi* yielded groundbreaking insights for the field of malaria research. For instance, scientists were able to show the basis for passive immunity in malaria by transferring serum of monkeys with chronic *P. knowlesi* infections (achieved by administering antimalarials early during infection) to freshly infected monkeys that were then able to recover (Coggeshall and Kumm, 1937). In later experiments, Coggeshall suggested that antibodies were responsible for this observed protective effect since antibodies in serum of human volunteers infected with *P. knowlesi* hindered disease progression in freshly infected monkeys (Coggeshall, 1940). Based on these and other results, the first vaccination experiments involving malaria parasites were performed: a study in which inactivated blood-stage *P. knowlesi* parasites (and adjuvants) had been injected into rhesus macaques (Freund *et al.*, 1945) was published alongside a similar study where avian malaria parasites were injected into ducks (Freund, Sommer and Walter, 1945). In both cases the immunizations prevented lethal outcomes of the infections.

The utilization, usefulness and potential of the *P. knowlesi* strain has been greatly expanded in the last almost 25 years. First, when it was adapted to continuous culture in cynomolgus RBCs (Kocken *et al.*, 2002) since it then became possible to perform long-term experiments, but culture conditions still necessitated access to a primate research facility. This changed when the strain was adapted to continuous culture in human RBCs in 2013 (Lim *et al.*, 2013; Moon *et al.*, 2013). This crucial innovation made it possible for a much larger number of laboratories and research facilities to use *P. knowlesi* for their experiments. For the adaptation, Moon *et al.* first initiated an *in vitro* culture from a *P. knowlesi* A1 strain stock frozen in 1976 (originally derived from the H strain) (Chin *et al.*, 1965), that had previously been maintained exclusively *in vivo* in macaques (Moon *et al.*, 2013) (Fig. 1.4). Since transferring the stock directly into 100% human RBCs had failed, they transferred the parasites into a 4:1 ratio of human to cynomolgus RBCs (Fig. 1.4). It was reasoned that 20% cynomolgus RBCs were sufficient to maintain the culture while the presence of 80% human RBCs gave parasites a selective advantage if they were able to invade and develop in human cells. After eight months of continuous culture, parasites were able to proliferate stably in 100% human RBCs, eventually with parasitemias increasing three- to fivefold daily. The resulting A1-H strain was then subcloned which yielded the A1-H.1 strain (Moon *et al.*, 2013) (Fig. 1.4) that is widely used today (Carrasquilla *et al.*, 2020; Ressurreição *et al.*, 2020; Ndegwa *et al.*, 2021) and is also the parental strain used in all experiments in this thesis. The genome of this strain has been fully sequenced (Benavente *et al.*, 2018). Interestingly, in this study by Moon *et al.*, it was found that the A1-H.1 strain retained the capacity to invade and develop in cynomolgus RBCs although it had been cultured exclusively in human RBCs for ca. one year before the respective experiments were performed. This finding opened the exciting possibility of combining *in vitro* and *in vivo* experiments in the future.

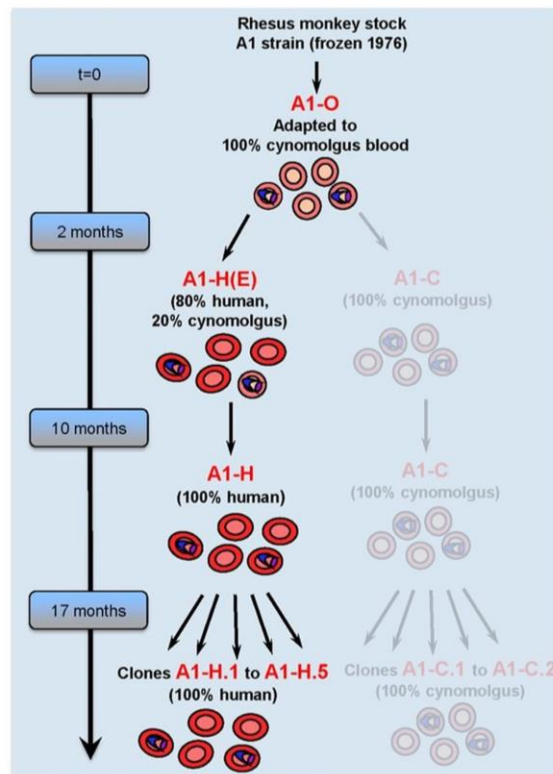


Fig. 1.4: Strategy for adapting *P. knowlesi* to continuous culture in human RBCs. *P. knowlesi* parasites of the A1 strain, taken from a rhesus monkey blood sample and frozen in 1976, were thawed and adapted to culture in 100% cynomolgus blood (strain A1-O; t=0). Over 2 months, the parasites were cultured in a mixture of 80% human and 20% cynomolgus RBCs (strain A1-H(E)). From there, over a span of 10 months, Moon *et al.* were able to adapt the parasites to 100% human RBCs (A1-H strain) and subsequently produced the clones A1-H-1 to A1-H.5 of the strain. At t=0, part of the culture was kept in 100% cynomolgus blood (strain A1-C) and cloned into A1-C-1 to A1-C.2 (greyed out). The figure was modified from (Moon *et al.*, 2013).

P. knowlesi has different invasion characteristics than *P. falciparum*, which is the only other human-infecting malaria parasite that has been adapted to continuous culture. In *P. knowlesi*, invasion into RBCs is mainly mediated by the interaction between the Duffy-binding protein (DBP) on the merozoite surface and the Duffy antigen receptor for chemokines (DARC) on human RBCs (Miller *et al.*, 1975; Singh *et al.*, 2002, 2005). In humans, the three most common allele types for the Duffy antigen (Fy) are Fy^A, Fy^B and F^{null} (Höher, Fiegenbaum and Almeida, 2018). Fy^A and Fy^B differ by a single nucleotide that leads to an amino acid substitution in the major subunit glycoprotein D (Tournamille, Le, *et al.*, 1995). The F^{null} allele is a silent Fy^B allele in which a single nucleotide substitution in the promoter prevents DARC expression specifically in erythroid cells (Tournamille, Colin, *et al.*, 1995). Interestingly, Moon *et al.* found that there was no preference for either the Fy^A or Fy^B phenotype in the newly adapted *P. knowlesi* strain. However, they observed that parasites cultured in RBCs from two donors had a markedly decreased multiplication rate and they therefore suspected that a heterozygous negative

phenotype ($Fy^A Fy^-$ and $Fy^B Fy^-$, respectively) was responsible for this (Moon *et al.*, 2013). This likely affects *P. knowlesi* normal cell culture in the lab which will be elaborated in the Discussion (see chapter 3.3).

1.3.2 Transfection as a bottleneck for functional investigation

The term transfection has been used when describing several different processes and procedures, depending on the context. In the context of this thesis, I will refer to transfection as the process in which (foreign) nucleic acids are introduced artificially into a (parasite) cell. While there are different methodologies to achieve transfection including chemical transfection, e.g. by calcium phosphate coprecipitation (Schenborn, 2000; Kumar, Nagarajan and Uchil, 2019), the following paragraph will focus on the physical method of electroporation (also called electroporabilization).

Transfection by electroporation is achieved by subjecting biological membranes to high-voltage electrical fields (Potter and Heller, 2018). These membranes act as electrical capacitors (they cannot pass the current) (Potter and Heller, 2018) so that, when the transmembrane voltage is increased from its usual state at ca. 0.1 V to 0.5-1.0 V, so called membrane “dimples” form (Kumar, Nagarajan and Uchil, 2019). It has been proposed that from these dimples hydrophobic pores form that can then, in turn, be stabilized into hydrophilic pores (Potter and Heller, 2018). It has been shown using mammalian cells that during transfection, the plasmid DNA is pushed towards the membrane side facing the negative electrode (Golzio, Teissié and Rols, 2002) and DNA aggregates form (Faurie *et al.*, 2010). Further experiments with mammalian cells have shown that most of the plasmid DNA enters the cell via endocytosis (Rosazza *et al.*, 2016) and that once the DNA is inside these cells, it is distributed and transported throughout the cytoplasm via the actin and microtubule network (Rosazza *et al.*, 2013).

The first instance electroporation has been used to transfect cells with DNA was in 1982 when Neumann *et al.* transfected mouse lymphoma cells with either circular or linear constructs coding for the herpes simplex thymidine kinase gene (Neumann *et al.*, 1982). Roughly a decade later, the first study reporting transfections in *Plasmodium* was published in which the authors achieved transient transfection of the chicken parasite *P. gallinaceum* gametocytes/zygotes (Goonewardene *et al.*, 1993). The fact that the researchers chose to transfect sexual stages for their ability to temporarily exist as exoerythrocytic forms alludes to one of the challenges of *Plasmodium* transfections: when parasites reside within RBCs (which they do for almost the entire asexual blood-stage) (see chapter 1.2) the DNA must cross multiple membrane layers (Goonewardene

et al., 1993). Accordingly, if the intraerythrocytic forms are to be transfected, plasmid DNA must first cross the RBC plasma membrane, then the parasitophorous vacuole membrane, followed by the parasite membrane (Wu *et al.*, 1995; Balu and Adams, 2007) before it reaches the parasite nucleus. The same principle of making use of forms that can be temporarily exoerythrocytic for transfection was applied when, a few years later, the first stable transfections in *Plasmodium berghei* were reported (van Dijk, Waters and Janse, 1995). Here, instead of sexual stages, the researchers used late-stage (segmented) schizonts under the assumption that the electrical pulse would lead to the release of (mature) merozoites which are then transfected, thereby making transfection more efficient (van Dijk, Waters and Janse, 1995; Balu and Adams, 2007). Almost in parallel, the first studies in which *P. falciparum* was transfected were published (Wu *et al.*, 1995; Crabb *et al.*, 1996; Wu, Kirkman and Wellems, 1996). The first transfections using *P. knowlesi* were published in 1997 (van der Wel *et al.*, 1997). In these experiments, the parasites were transfected with constructs that included the selectable marker dihydrofolate reductase thymidylate-synthase that confers resistance to the drug pyrimethamine, either from *Toxoplasma gondii* or *P. berghei*. Using control regions from either *P. berghei* or *P. falciparum* for the expression of the resistance marker they found that both were active in *P. knowlesi*. As mentioned above, when using the intra-erythrocytic parasite forms for transfection, plasmid DNA must cross three different barriers before it reaches the parasite nucleus. This is thought to render the process very inefficient, as demonstrated by a transfection efficiency of ca. 10^{-6} in the *P. falciparum* strain when this form of transfection is performed (O'Donnell *et al.*, 2002; Balu *et al.*, 2009; Carrasquilla *et al.*, 2020). Since the ability to perform efficient transfections is so crucial for investigating genes of unknown function, such studies have previously been greatly hampered by the poor transfection efficiency in *Plasmodium*. This inefficiency dictated the use of great numbers of parasites which, in turn, meant using massive amounts of resources (human blood or animal hosts) and put especially large-scale investigations out of reach in the past (de Koning-Ward, Gilson and Crabb, 2015).

While it could be argued that there have not been many major technological advancements concerning transfections in *Plasmodium* parasites over the last years and decades, there are a few exceptions. For example, in 2006, Janse *et al.* published a study showing a massive increase in transfection efficiency in *P. berghei* parasites (Janse *et al.*, 2006). They combined using segmented schizonts for transfection with the Amaxa electroporation system called nucleofector which increased the efficiency from 10^{-9} - 10^{-7} for the standard protocol at that time to 10^{-7} - 10^{-3} , with optimal reproducible efficiencies of up to 10^{-3} . Several years later, Moon *et al.* applied the same principle to the *P. knowlesi* strain that they had just adapted to cell culture (A1-H.1) and were

able to achieve an unprecedented transfection efficiency of 30-40% (Moon *et al.*, 2013). The researchers estimated that this efficiency is at least 1000x higher than in *P. falciparum* (Moon *et al.*, 2013) and it is still the highest transfection efficiency ever published in a *Plasmodium* strain. For these transfection experiments, Moon *et al.* developed a plasmid, called pPkconGFP, which they based on a construct that had been commonly used for *P. falciparum* transfections (Reed *et al.*, 2000; Moon *et al.*, 2013). They exchanged regulatory sequences to *P. knowlesi*-specific sequences which drive the expression of the fluorescent marker enhanced green fluorescent protein. This plasmid was used to measure transfection efficiency in this publication by the Moon lab (Moon *et al.*, 2013) and in this thesis (see chapters 2.2.1 and 4.1.8).

Although this makes *P. knowlesi* a very promising strain for experiments in which large-scale transfections are required, unfortunately, there have been only a few studies that investigate the process of transfection itself in *P. knowlesi* (Vieira, Astro and Moraes Barros, 2021).

1.4 Recent advances in genome editing and its applications in *Plasmodium*

Generally, advances in genome editing in *Plasmodium* have closely followed genome editing advances in other fields of biological research in the past. Accordingly, first attempts at altering genomic sequences in a targeted way in the parasite relied on what we now call classical or conventional homologous recombination (Crabb *et al.*, 1996; Wu, Kirkman and Wellem, 1996). This form of genome editing essentially makes use of the capacity of homologous recombination to reliably repair DNA damage, including DSBs, which can arise from a variety of endogenous or exogenous sources (Li and Heyer, 2008). By providing a template that contains sequences homologous to the genomic locus that is supposed to be altered, researchers were able to exchange/recombine these sequences via single- or double crossover recombination in *Plasmodium* (de Koning-Ward, Gilson and Crabb, 2015).

The next step was taken by developing more sophisticated approaches in which nucleases were used to cut the target locus at specific sequences to increase the frequency of homologous recombination. One type of these nucleases are so-called homing endonucleases that are very specific DNA-cutting enzymes, naturally occurring and ubiquitously prevalent in microbes (Stoddard, 2011) (Fig. 1.5 C). Nucleases belonging to a different type are engineered enzymes based on the FokI nuclease domain, such as zinc finger nucleases and transcription activator-like enhancer nucleases (Fig. 1.5 C and D). Zinc finger nucleases were also adapted to the parasite so that it was, for example, possible to obtain gene knockout parasites faster than ever

before (Straimer *et al.*, 2012). However, using this methodology, engineering a new nuclease for each target rendered the experimental process time- and resource-consuming (Zhu, 2022).

This fundamentally changed when the arguably most important scientific and technological advancement regarding genome editing in the recent past emerged, namely the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated genes (Cas) (CRISPR-Cas) system (Fig. 1.5 G, here exemplary shown with Cas9 as nuclease).

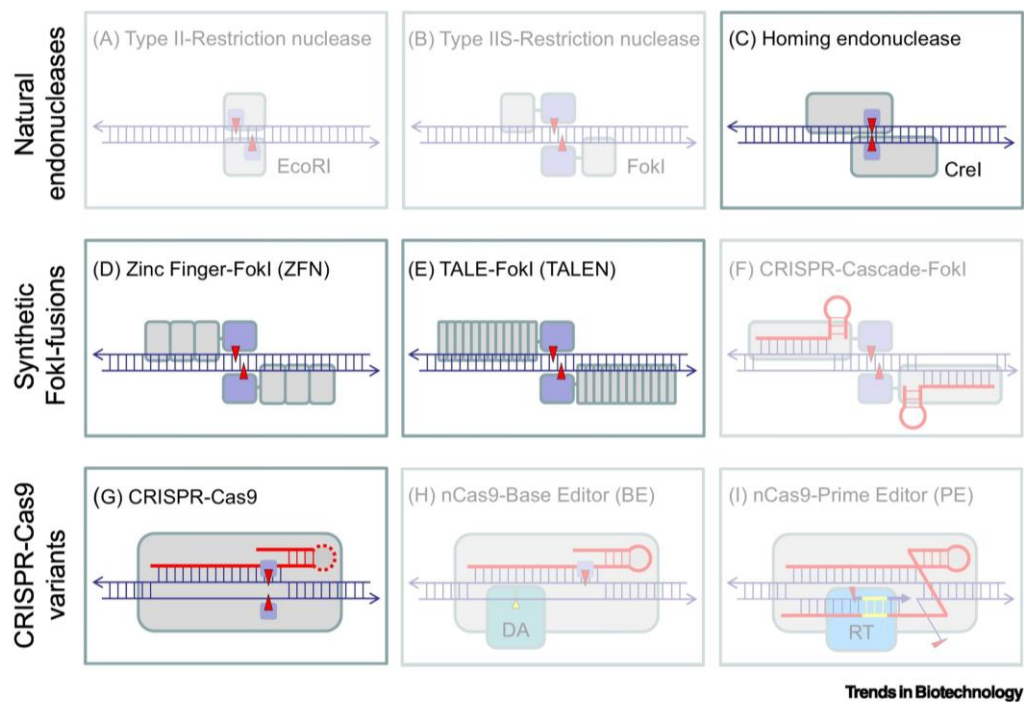


Fig. 1.5: Overview of different types of nucleases that have been used for genome editing. While homing endonucleases (C) have been used in their natural form, some endonucleases have been artificially engineered by introducing the FokI domain that confers target specificity. This includes zinc finger nucleases (D) and transcription activator-like enhancer nucleases (E). Through the discovery of CRISPR, a range of associated nucleases and variants have been developed, including the canonical CRISPR-Cas9 (G). Panels showing nucleases not pertaining to this thesis ((A), (B), (F), (H) and (I)) have been greyed out. The figure was modified from (van der Oost and Patinios, 2023).

This system was originally identified to be a form of adaptive immune defense system in bacteria and archaea against viral attack (Barrangou *et al.*, 2007; Jinek *et al.*, 2012) and has developed in several variations in different microbes (Makarova *et al.*, 2011; Mojica and Rodriguez-Valera, 2016).

The common principle among them is that after foreign DNA has been introduced into the cell (e.g., viral attack or encounter with mobile genetic elements), the microbe stores a short

sequence of that DNA (also called “spacer”) in a designated locus (i.e., the CRISPR locus) with repeats dividing the different spacers (“adaptive phase”, Fig. 1.6 A “1st stage”) (Makarova *et al.*, 2011) . During the so-called “expression phase” (Fig. 1.6 A “2nd stage”) this CRISPR locus is transcribed and the precursor CRISPR RNA is processed so that it can associate with an effector endonuclease during the “interference phase” (Fig. 1.6 A “3rd stage”) that will, in turn, cleave the respective foreign DNA element upon challenge (Makarova *et al.*, 2011).

After the discovery of this intriguing system, scientists have realized its great potential for applications in genome editing, adapted it for use in many different organisms (Cong *et al.*, 2013; DiCarlo *et al.*, 2013; Gratz *et al.*, 2013; Hwang *et al.*, 2013; Jiang *et al.*, 2013; Jinek *et al.*, 2013; Mali *et al.*, 2013) and developed it further during the last decade. The most common form of CRISPR-Cas employs a Cas9 endonuclease from *Streptococcus pyogenes*, which is a class 2/type II Cas protein (Koonin and Makarova, 2019), meaning it is a single multidomain protein instead of a complex of several proteins. Many, especially newer, applications have used an engineered chimeric single guide RNA (sgRNA) in which a hairpin was fused to the CRISPR RNA (crRNA) to mimic the trans-acting crRNA (tracrRNA) for binding Cas9 (Jinek *et al.*, 2012; Qi *et al.*, 2013) (Fig. 1.6 B). The sgRNA establishes contact with the target via Watson-Crick base-pairing whereupon two domains of Cas9 with endonuclease activity make a DSB 3 nucleotides upstream of a sequence motif called protospacer adjacent motif (PAM) (Lander, 2016) (Fig. 1.6 C). The PAM is a prerequisite for successful targeting of CRISPR-Cas and the PAM of *Sp*Cas9 is -NGG (Mojica and Rodriguez-Valera, 2016), where “N” can be any nucleotide.

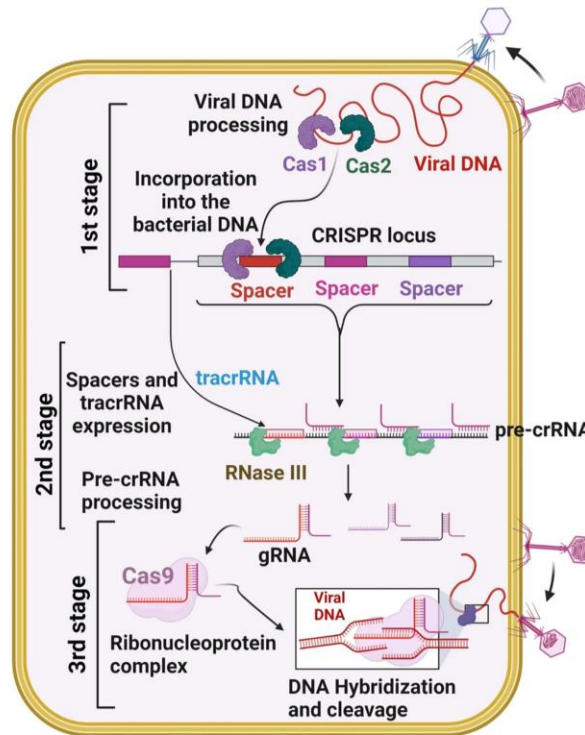
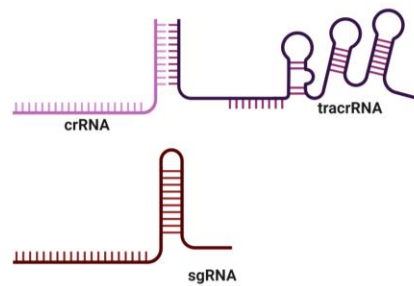
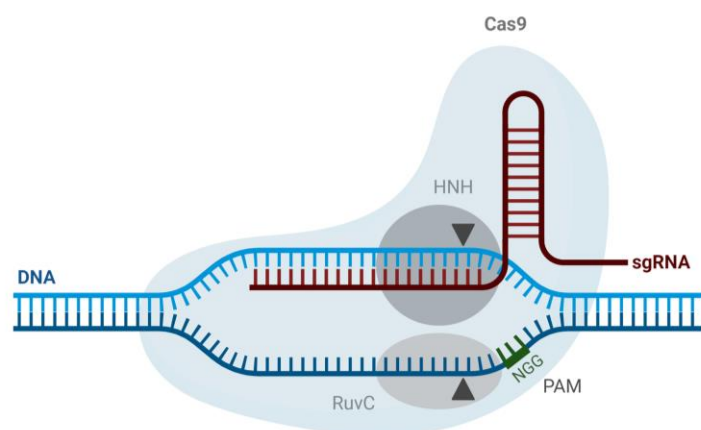
A**B****C**

Fig. 1.6: The CRISPR-Cas system. (A) The CRISPR-Cas system was originally identified to act as a form of adaptive immune system in a wide range of bacteria and archaea. Upon detection of foreign DNA in the cell, a portion of the DNA is stored in the CRISPR locus as spacers, interrupted by repeats (1st stage). Expression of the CRISPR locus yields a pre-CRISPR RNA (pre-crRNA) that is processed by RNase III yielding mature guide RNA (gRNA) (2nd stage). Upon second entry of a particular foreign DNA, a complex of gRNA and Cas endonuclease can bind the targeted DNA by hybridization mediated by the gRNA and the DNA is cleaved (3rd stage). This panel

was taken from (Hillary and Ceasar, 2023). (B) Originally, a gRNA consisting of a crRNA and a tracrRNA (top) has been used. Most applications today use a simplified version, the single guide RNA (sgRNA), where a hairpin was fused to the spacer-portion of the gRNA which is required for binding to Cas9. (C) Upon binding of the sgRNA to Cas9, the complex is recruited to its target by hybridization of the spacer portion of the sgRNA with the protospacer of the target locus. The endonuclease domains HNH and RuvC together introduce a double strand break 3 nucleotides upstream of the PAM. (B) and (C) were created with BioRender.com.

In many organisms DSBs are repaired via non-homologous end joining (NHEJ) which regularly leads to insertion-deletion frameshift mutations and therefore leads to a knockout of the gene in question (Song *et al.*, 2021). It has been shown that the canonical NHEJ pathway does not exist in *Plasmodium* and DSBs are rather repaired via homology-directed repair if a homologous template is available (Kirkman, Lawrence and Deitsch, 2014) or alternative NHEJ pathways to a lesser degree (Kirkman, Lawrence and Deitsch, 2014; Singer *et al.*, 2015).

Compared to alternative techniques mentioned above, CRISPR-Cas9 has several advantages. As mentioned above, both zinc finger nucleases and transcription activator-like enhancer nucleases needed to be redesigned for each gene target which entailed laborious and time-consuming cloning. With CRISPR-Cas9, the specificity of editing comes from the 20-nucleotide long spacer portion of the sgRNA so that the endonuclease becomes essentially programmable by designing a short oligonucleotide which greatly reduces time, effort, (Zhu, 2022) and thereby also financial resources per gene target. CRISPR-Cas9 has also shown an unprecedented versatility of application. Including, but not limited to, the possibility to use multiplex approaches where several targets are altered at the same time (Cong *et al.*, 2013), as well as modifications of Cas9 where one or both of its nuclease domains have been inactivated, rendering the nuclease with either nickase activity (Cong *et al.*, 2013) (cleaving only one DNA strand) or catalytically inactive (dCas9; see chapter 1.6).

It has been a decade since the CRISPR-Cas9 system was established in *Plasmodium* parasites (Ghorbal *et al.*, 2014) and it has set itself apart as the method of choice for genome editing in this organism. Using CRISPR-Cas9, researchers have been able to genetically engineer *Plasmodium* parasites without introducing a marker which was already implemented in the work that used CRISPR-Cas9 in *Plasmodium* for the first time (Ghorbal *et al.*, 2014). This alleviated the challenge of scarce selectable markers that researchers in this field had to face previously. In fact, by using this technology it was even possible to perform “scar-less” editing (Walker and Lindner, 2019; Liang *et al.*, 2022) for the first time, which means that, besides the desired sequence alterations, no other changes are left at the targeted genomic locus.

A range of optimization measures, both in general and parasite-specific, have further advanced this technology. Generally, successful targeting and editing by CRISPR-Cas9 relies on how

efficient the sgRNA binds its respective complementary sequence of the target locus. Through systematic studies researchers have found that the efficiency of sgRNAs depends on several different parameters and have tried to formulate rules which, in turn, informed tools that, e.g., score the quality of spacer sequences, to help design the most efficient sgRNAs possible in each scenario (Doench *et al.*, 2014, 2016; Labuhn *et al.*, 2018). These studies were performed in mammalian/human cells and since researchers have found that there seem to be differences regarding these rules in parasites, there have been efforts to adapt these rules and tools, respectively, to *Plasmodium* parasites (MacPherson and Scherf, 2015; Peng and Tarleton, 2015; Ribeiro *et al.*, 2018).

A very interesting innovation has been developed by Walker and Lindner (2019), namely a ribozyme-mediated multiplex CRISPR editing approach. Here, they used a ribozyme-guide-ribozyme (RGR) array which not only allows the expression of either single or multiple sgRNAs at the same time (multiplex) but also to use an RNA polymerase II binding/attracting promoter instead of a promoter for RNA polymerase III (Fig. 1.7). This opens the possibility to use a lot more transcriptional regulation, e.g., conditional expression, including stage-specific expression (Walker and Lindner, 2019).

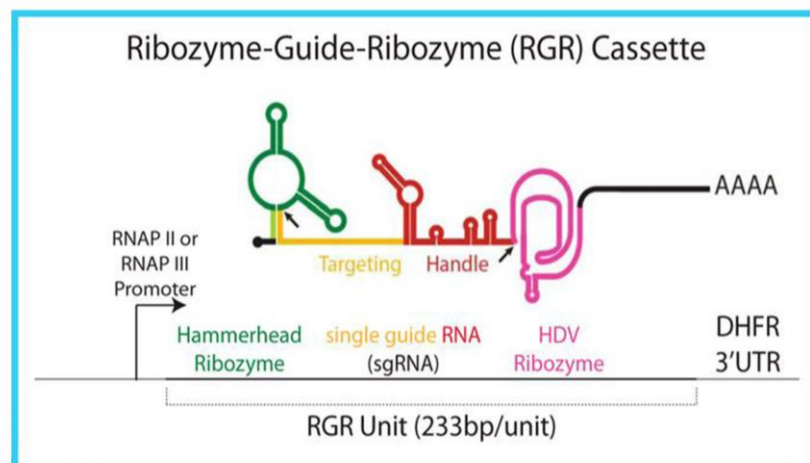


Fig. 1.7: The ribozyme-guide-ribozyme (RGR) system for expression of sgRNAs developed by Walker and Lindner. In this system, Walker and Lindner built an expression cassette where the sgRNA sequence (yellow and red) is flanked by the sequences coding for hammerhead ribozyme (green) and hepatitis delta-virus ribozyme (pink). The addition of the ribozymes allow expression of the sgRNA cassette not only by RNA polymerase III (as is common for RNA-expressing constructs) but also from RNA polymerase II which opens the possibility for e.g., stage-specific expression. The precise self-cleavage by the ribozymes (indicated by black arrows) ensures that only the sgRNA remains without any added nucleotides. This figure was modified from (Walker and Lindner, 2019).

1.5 Genome-wide screening approaches in *Plasmodium*

Genome-wide screens have been crucial in annotating the functional genome of many species and organisms (Grimm, 2004; Boutros and Ahringer, 2008), including important model organisms, such as yeast (Forsburg, 2001), zebrafish (Patton and Zon, 2001) or the nematode *Caenorhabditis elegans* (Jorgensen and Mango, 2002).

Although the genomes of *P. falciparum* and *P. knowlesi* had already been published in 2002 and 2008, respectively (Gardner *et al.*, 2002; Pain *et al.*, 2008), the endeavor of discovering novel gene function in a large-scale format in *Plasmodium* parasites has been met with extraordinary challenges (Ishizaki *et al.*, 2022). One such challenge is that the biology of *Plasmodium* parasites is quite divergent from vertebrates or even from other unicellular organisms, such as common model organisms. Therefore, it has been extremely difficult to predict the function of genes that are unique to these parasites (Sexton *et al.*, 2019). Another challenge is the extremely high AT-content of the *Plasmodium* genome. In the *P. falciparum* strain, the AT-content averages 80.6% overall and reaches an even higher content of ca. 90% in intergenic regions and untranslated regions (UTRs) (Gardner *et al.*, 2002), which makes molecular cloning of targeting vectors difficult (Wu *et al.*, 1995; Carrasquilla *et al.*, 2020). Another challenge concerns the way these vectors are transferred into the parasites, namely the already mentioned low transfection efficiency of *Plasmodium*, especially of *P. falciparum*, which amounts to only 10^{-6} (O'Donnell *et al.*, 2002; Balu *et al.*, 2009; Carrasquilla *et al.*, 2020). This has made large-scale studies virtually impossible or at least very impractical in the past (de Koning-Ward, Gilson and Crabb, 2015).

In 2018, Zhang *et al.* published the first saturation-level genomic screen in *Plasmodium* (Zhang *et al.*, 2018). Here, they used the *piggyBac* transposon for mutagenesis which is a transposable element derived from the cabbage looper *Trichoplusia ni* and emerged as a suitable tool for this application for two reasons. Since it belongs to the class II transposons which exclusively targets TTA sites it, firstly, elegantly exploited the genome's excessive AT-content and, secondly, always inserts and excises in a precise manner (Balu *et al.*, 2005). In total, Zhang *et al.* were able to identify 38,000 independent *piggyBac* insertions targeting 5399 nuclear protein-coding genes across all 14 chromosomes of *P. falciparum* NF54 strain parasites (Fig. 1.8 A), even exceeding the number of insertions they had estimated to be required for a saturation-level screen by ca. 5000 insertions (Zhang *et al.*, 2018). They counted the number of insertions by quantitative insertion-site sequencing which they had previously validated for *piggyBac* screening in *P. falciparum* (Bronner *et al.*, 2016). The uniform distribution of insertions throughout and across all chromosomes suggested that the interpretation of the results would not be biased by chromatin structure or locations of the genes. Analysis of the data revealed that 3357 genes did

not have any insertions (Fig. 1.8 B, “nonmutable”), of which 12% received a tentative classification as essential because (i) the genes contained no TTAA sites or fewer than could be expected from their gene length or (ii) because the genes fell below the threshold of 500 bp in length (Zhang *et al.*, 2018). This left 2680 genes, i.e., 49.6% of the genes targeted in the screen, which were identified to be essential.

Zhang *et al.* calculated two scores to assess the essentiality status and fitness of the *P. falciparum* genes targeted in their screen. One is the mutagenesis index score (MIS) which defines the mutability of a certain *P. falciparum* gene as determined by the *piggyBac* insertions or lack thereof (Fig. 1.8 C). The other one is the mutagenesis fitness score (MFS), that, although being independent, correlated well with the MIS and rates the growth phenotype of parasites in which the gene in question is mutated. This way, they were able to classify the mutability and fitness cost of mutations of 87% of all *P. falciparum* genes. Although Zhang *et al.* were able to exploit the very high genomic AT-content of *P. falciparum*, they were still faced with its poor transfection efficiency, so that immense numbers of parasitic culture had to be used for this study: parasites were transfected in a 96-well plate format with 10 million schizont-stage parasites per plate and 100 plates transfected in total.

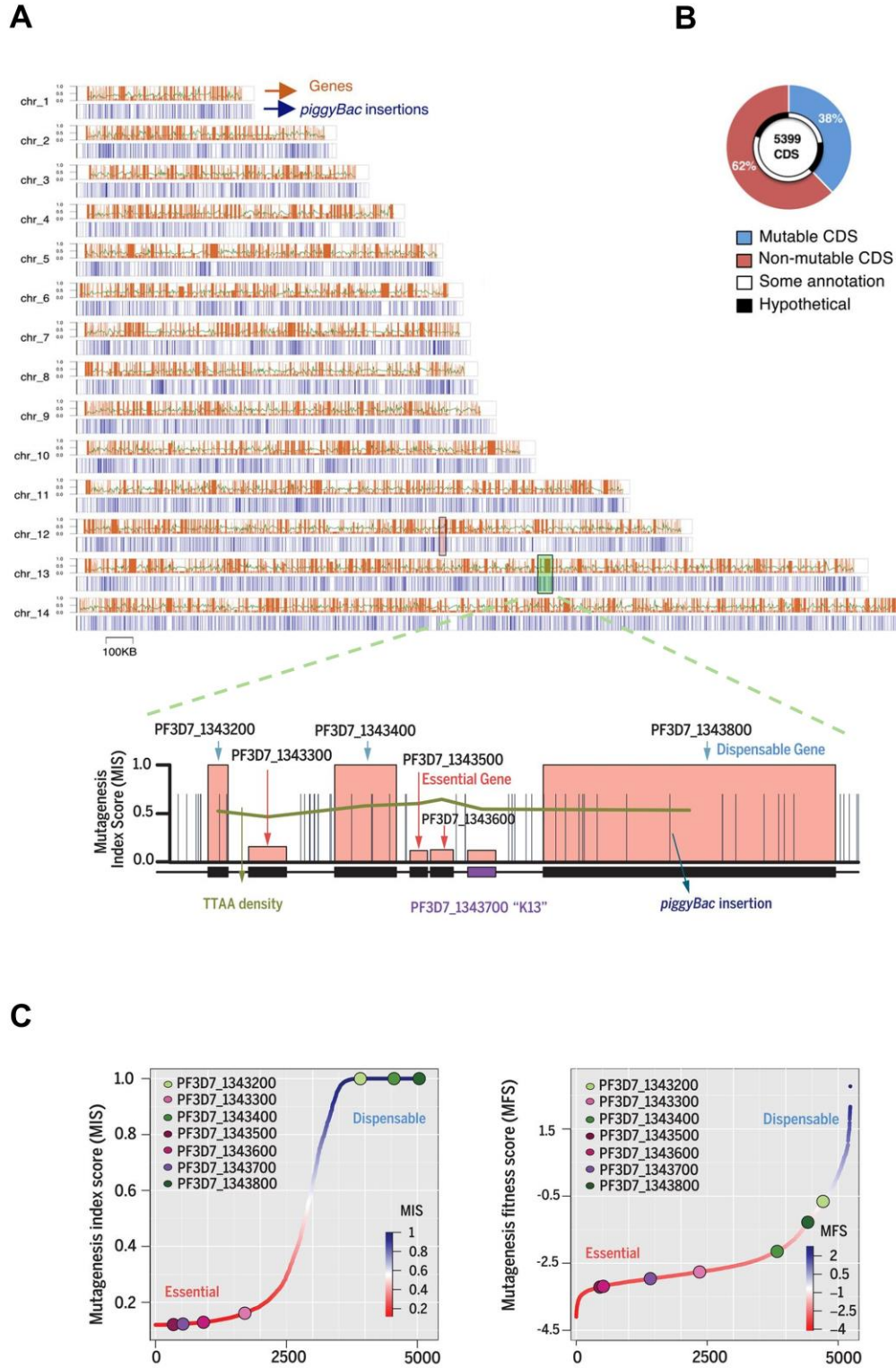
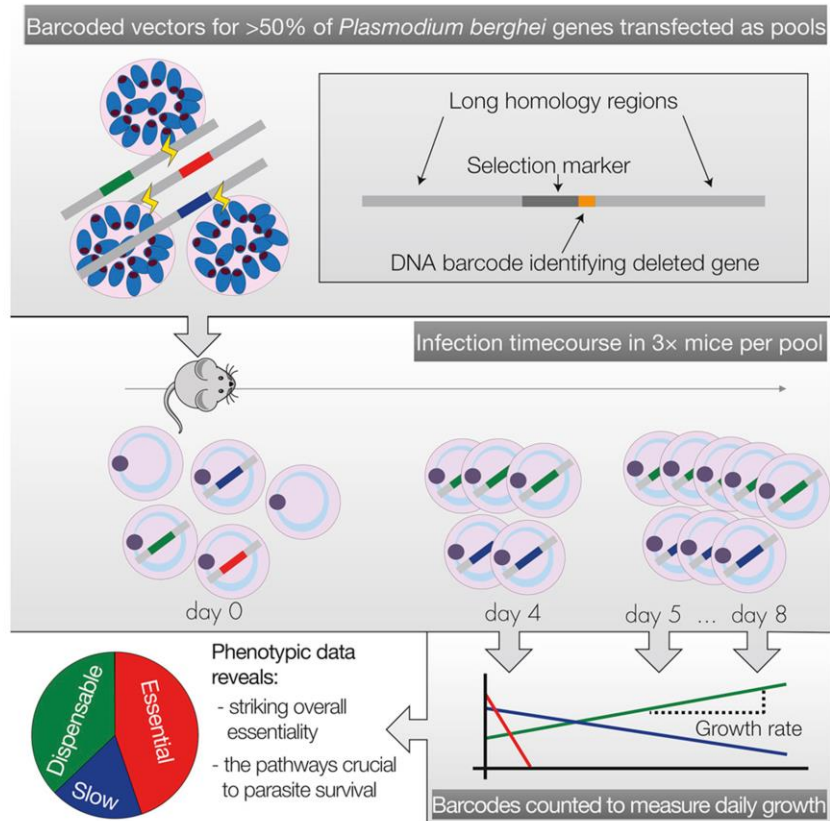


Fig. 1.8: Saturation mutagenesis of the *P. falciparum* genome during asexual blood-stage growth. (A) Visual representation on the top shows uniform distribution of the 38,000 *piggyBac* insertions (blue) within and across all 14 chromosomes of *P. falciparum*. Genes are shown in orange. The green box on chromosome 13 is zoomed in on the bottom and shows a graph depicting the Mutagenesis Index Score (MIS; y-axis) calculated for genes in an exemplary locus, including the known essential gene *PfK13* (coding sequence (CDS) represented by purple box). The higher the MIS of a gene's CDS (light red box), the higher the propensity of the respective gene to be dispensable, as illustrated by *piggyBac* insertions (thin dark vertical lines) retrieved from that gene sequence.

(B) Of 5399 genes targeted by this screen, the majority (62%) had non-mutable coding sequences whereas 38% were mutable (outer ring). For both of those groups, the majority (white, inner circle) have some annotation compared to hypothetical annotations (black). (C) To assess the results of the screen, Zhang *et al.* devised two independent scores: the MIS (left) rates the potential mutability of targeted genes based on the number of *piggyBac* insertions they found in relation to how many insertions would be expected (based on e.g. gene length). The mutagenesis fitness score (MFS; right) rates the relative fitness a given gene confers on the parasite based on a score they got from the sequencing process that identified the insertion sites. In both cases, genes with known essentiality status are depicted as validation of the score: genes essential for *in vitro* blood-stage growth have low MIS and MFS, respectively, and conversely, dispensable genes under these conditions have high scores. The figure was modified from (Zhang *et al.*, 2018).

Interestingly, a year prior, another large-scale screen in *Plasmodium*, targeting >50% (2578 genes in total) of the *P. berghei* genome, reported a similar proportion of 44.9% genes essential for *in vivo* growth in mice (Bushell *et al.*, 2017). This approach was made possible through a massive collaborative effort by the so-called **Plasmodium genetic modification (PlasmoGEM)** project, which is an open access and not-for-profit initiative. With a concerted effort it was possible to produce the PlasmoGEM knock-out vectors one-by-one that were used for this study. Here, Bushell *et al.* determined a relative growth rate for each mutant parasite by counting a unique barcode, which is part of the PlasmoGEM disruption vectors, in daily blood samples using a next generation sequencer (Fig. 1.9 A). This way, they differentiated between four different types of growth behavior of the mutants: no growth (target gene essential), slow growth, normal growth (target gene dispensable) and fast growth. The latter category consisted of only two genes: the transcription factor known as the master regulator of gametocytogenesis AP2-G as well as a likely false positive hit. In addition to the ca. 45% genes classified as essential, they also found that the mutants of further 18.0% of genes exhibited slower growth compared to the controls and WT parasites, amounting to ca. 2/3 of the genome being required for normal blood-stage growth (Fig. 1.9 B). The authors compared this proportion of essential genes to functional gene annotations in other organisms, including well-known model organisms, e.g., *Saccharomyces cerevisiae* (Giaever *et al.*, 2002; Deutschbauer *et al.*, 2005), but also other *Apicomplexan* parasites, e.g., *Toxoplasma gondii* (Sidik *et al.*, 2016) and *Trypanosoma brucei* (Alsford *et al.*, 2011) (Fig. 1.9 B). This revealed that the 62.9% of genes required for normal growth in *P. berghei* is surprisingly high compared to ca. 35-40% in these other species (Bushell *et al.*, 2017). In their paper, Bushell *et al.* explored the relationship between this phenomenon and evolutionary forces acting on the parasite's genome, such as adaptations to the parasite's lifestyle that was accompanied by the loss of thousands of genes (Bushell *et al.*, 2017). Hence, they suggested that these events could be responsible for the parasite's compact genome with such a high proportion of genes contributing to normal growth in just one of several complex life cycle stages of *Plasmodium*.

A



B

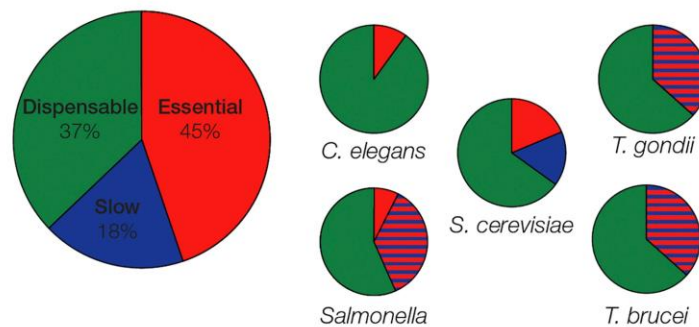


Fig. 1.9: A large-scale screen targeting more than half of *P. berghei*'s genome identifies an unexpectedly high proportion of genes required for normal asexual blood-stage growth. (A) Graphical summary of the screen. *P. berghei* parasites were transfected in pools with *Plasmo*GEM knockout vectors that included a barcode sequence for target identification. After transfection, the parasite pools were transferred into mice and samples were taken daily to monitor parasite growth in 3 mice per plasmid pool. The relative abundance of barcodes in each sample was measured with next generation sequencing and a corresponding growth rate was calculated. (B) According to the growth rates of the mutant parasites Bushell *et al.* categorized the corresponding genes as essential, slow (growth) or dispensable. They found an unusually high percentage of essential genes (45%), especially when combined with genes that if targeted yielded slow growth (18%), in the *P. berghei* genome compared to the genome

of other species. These included some commonly used model organisms (*C. elegans* and *S. cerevisiae*) but also other *Apicomplexan* parasites (*T. brucei* and *T. gondii*). This figure was modified from (Bushell *et al.*, 2017).

Both screens were large-scale forward genetic approaches that were made possible, in part, by technological advancements, such as next generation sequencing, which allowed the identification of gene hits based on the phenotype they produced. Due to the relatively inefficient transfection of *Plasmodium*, massive amounts of parasites had to be cultivated and transfected in batches (e.g., 58 pools of transfecting ca. 100 *Plasmo*GEM vectors) to reach the screening coverage of >50% of genome and genome-wide for the *P. berghei* and *P. falciparum* screens, respectively.

It is also due to the large-scale nature of the screens that they proved to be of high importance and value to the scientific community. The screen using *Plasmo*GEM vectors raised the number of *P. berghei* genes with any functional annotation available by over 5-fold (Bushell *et al.*, 2017) and the relative growth rate data of the mutants was made available on the website of the *Plasmo*GEM project (<https://plasmogem.umu.se/pbgem/>). Similarly, the MIS and MFS data from the Zhang *et al.* screen was also made available and can be accessed via the *Plasmodium* Informatics Resource PlasmoDB (<https://plasmodb.org/plasmo/app/>). These resources can act as the starting point to help prioritize novel drug targets, especially candidates that play important roles in multiple life cycle stages, or cellular processes that could be explored for therapeutic intervention. In addition, they can also give perspective to ongoing studies, e.g., most blood-stage vaccine candidates in *P. falciparum* showed a dispensable phenotype when mutated except for reticulocyte binding protein homologue 5. Both screens complement each other well in this regard since the screen with the higher coverage used *P. falciparum* cultures grown *in vitro* while the mutant *P. berghei* parasites in the *Plasmo*GEM screen were grown *in vivo*.

As relevant as these studies were for the malaria research field, their most important limitation was that both screens employed knockout approaches, which, by definition, exclude essential genes from further functional annotation within the screen. Thus, over 20 years after the publication of the *P. falciparum* genome, its overall functional annotation has progressed slowly. In the newest version of the *P. falciparum* 3D7 genome (version 3.2 from 2019), of 5438 genes identified in total (including pseudogenes) 1776 genes (32.7%) still remain without any functional annotation, 2209 (40.6%) genes have a putative function and only 1296 genes (23.8%) have experimental evidence for their function (Böhme *et al.*, 2019). This highlights the need for more efficient and rapid course of action regarding gene function analyses.

1.6 CRISPR interference (and activation) technologies

As indicated in chapter 1.4, the CRISPR interference or activation technologies are derived from the type II CRISPR-Cas system which has a single Cas effector nuclease. In most cases, Cas9 from *S. pyogenes* is utilized (Lee *et al.*, 2019), which likely makes it the best characterized Cas protein to date. In contrast to the canonical CRISPR-Cas system, the endonuclease domains RuvC and HNH in Cas9 are mutated (D10A in RuvC and H840A in HNH) so that the Cas protein is rendered catalytically inactive or “dead” – hence, the abbreviation dCas9 (Qi *et al.*, 2013) (Fig. 1.10). Upon binding of a sgRNA to dCas9, the resulting dCas9-sgRNA complex is guided to the respective target sequence and the complex then binds, but it does not cleave the DNA. To use this for interfering with gene expression, the dCas9 can be targeted to regions upstream of the CDS of a gene to prevent or interfere with binding of the RNA polymerase (Fig. 1.10) or transcription factors, or downstream of the translation start to interfere with transcription elongation (Qi *et al.*, 2013).

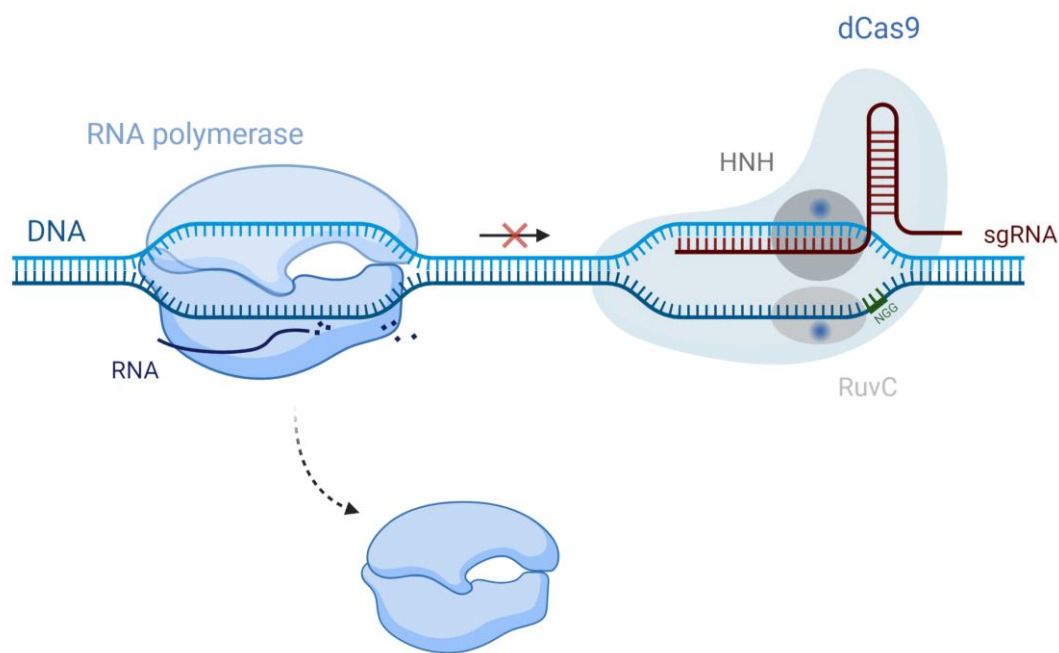


Fig. 1.10: CRISPR interference employs sgRNA-programmable dCas9 to interfere with target gene expression. CRISPR interference (CRISPRi) utilizes a catalytically inactive or “dead” mutant of Cas9 (dCas9) where its endonuclease domains HNH and RuvC have been mutated through point mutations (D10A in RuvC and H840A in HNH) (blue circles). Therefore, the dCas9 that has been recruited to a target locus via sgRNA cannot cleave the DNA but instead interferes with the binding of factors necessary for gene expression, especially RNA polymerase. This panel was created with BioRender.com

While this approach has been shown to work successfully in a variety of organisms, the CRISPRi toolbox has been quickly expanded to couple the dCas9 with effector domains in yeast and

human cells (Gilbert *et al.*, 2013). This way it is not only possible to increase the downregulation effect of the dCas9-mediated knockdown by fusing dCas9 to a repressive domain (e.g., KRAB-domain), dCas9 can also be fused to a transcriptional activator domain (e.g., VP64-domain) to upregulate expression of a target gene (Gilbert *et al.*, 2013).

Since the protospacer adjacent motif (PAM) of Cas9 is so short (-NGG), a multitude of possible sgRNAs are available to target a given gene but not all of them will lead to an efficient downregulation (or upregulation) of the target. Therefore, Gilbert *et al.* set out to determine “rules” for the design of efficient sgRNAs. Using a dCas9-KRAB fusion protein, they systematically examined all unique sgRNAs in a certain region upstream of 49 genes in human cells that are known to be associated with sensitivity to the toxin ricin (Gilbert *et al.*, 2014). By tiling the transcription start sites (TSSs) of these 49 genes with sgRNAs, they determined which sgRNAs were responsible for the most extreme changes in gene expression when cells were grown in absence or presence of the toxin. Accordingly, they deduced that CRISPRi was efficient in human cells when sgRNAs targeted regions of -50 to +300 bp around the genes’ TSSs, with maximal interference when bound +50 to +100 bp of the TSS (Gilbert *et al.*, 2014).

Coinciding, the first study showing CRISPRi in *Plasmodium* also used chimeric forms of dCas9 where it was either fused to the histone acetyltransferase domain GCN5 or the histone deacetylase domain Sir2a (Xiao *et al.*, 2019) (Fig. 1.11 A and B). In this work, the researchers demonstrated the capacity of their system to activate transcription of a silent gene as well as to downregulate two other endogenous genes in the *P. falciparum* genome. They targeted the erythrocyte invasion-related gene EBA-175 and the histone methyltransferase gene SET1 and measured a significant reduction in transcripts for both by RT-qPCR. They used the Dd2 strain of *P. falciparum* in which the gene RH4 is silent. By employing their dCas9-GCN5 fusion construct they found a 113-fold increase in RH4 transcripts by RT-qPCR. To validate the specificity of these results, they used chromatin immunoprecipitation quantitative polymerase chain reaction (ChIP-qPCR) with FLAG antibodies and were able to show FLAG-tagged dCas9 occupancy at the TSS of RH4, EBA-175 and SET1. In all three cases, the researchers showed results with 1-2 sgRNAs targeting the respective genes.

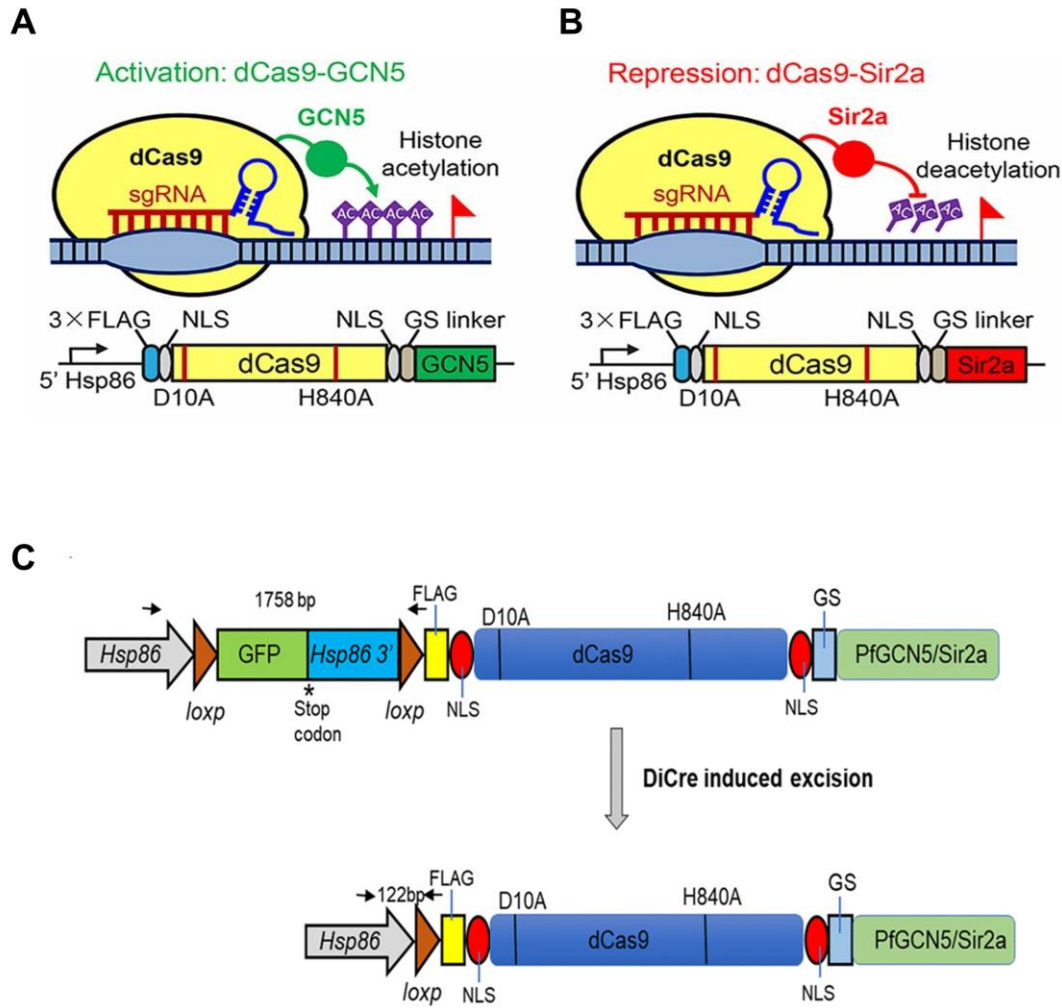


Fig. 1.11: CRISPR interference systems using dCas9 fusion proteins in *P. falciparum*. (A) and (B) show the schematic of the CRISPR interference systems. In (A), dCas9 was fused to the histone acetyltransferase domain GCN5 (green structures), whereas in (B) it was fused to the histone deacetylase domain Sir2a (red structures). These mediate transcriptional activation and repression of the target gene, respectively. The transcription start site of the gene is indicated by a red flag. The panels in (A) and (B) were modified from (Xiao *et al.*, 2019) (C) The system shown in (A) and (B) was developed further to become inducible using the DiCre/loxP system. A GFP sequence including a stop codon at the end and a Hsp86 3' UTR were inserted upstream of the dCas9 expression cassette, flanked by two loxP sites. In the absence of rapamycin, only this GFP cassette is expressed. Upon addition of rapamycin, DiCre excises the GFP cassette via the loxP sites and the respective dCas9 fusion product is expressed. This panel was modified from (Liang *et al.*, 2022).

Another study, that was published later the same year, used dCas9 without effector domains in the rodent malaria parasite *P. yoelii* (Walker and Lindner, 2019). The researchers had developed a system based on canonical CRISPR-Cas which facilitated the expression of several sgRNAs from the same construct using ribozymes and sought to expand this technology to CRISPRi. They used an endogenous locus that they had previously tagged with green fluorescent protein (GFP) (*pyalba4::gfp*) and targeted this with 11 sgRNAs in addition to one control sgRNA. Here they found a 2-3-fold reduction in GFP mean fluorescence intensity for some of the sgRNAs.

Interestingly, when they examined the target region of the two most potent sgRNAs, they found the core motif for AP2-G which is an important transcription factor in *Plasmodium* (Sinha *et al.*, 2014). Of note, when they expressed several sgRNAs from the same plasmid (either two or ten simultaneously) they found that the knockdown effect was not stronger compared to expressing only one sgRNA (Walker and Lindner, 2019). They also observed that there was no difference in knockdown efficiency depending on which DNA strand was targeted. Although there seem to be conflicting results regarding that (Wang *et al.*, 2014), the result is in line with previous studies that reported no strand-specific effect when regions upstream of the CDS were targeted in *Escherichia coli* as well as in murine and human cells (Qi *et al.*, 2013; Doench *et al.*, 2014; Gilbert *et al.*, 2014). Complementary, when sequences in the CDS are targeted, Ribeiro *et al.* hypothesized that, at least in mammalian cells, sgRNAs might be ineffective due to unzipping of the effectors by RNA polymerase (Ribeiro *et al.*, 2018).

There have also been studies published that used a CRISPRi approach employing a dCas9 without effector domains in *P. falciparum* (Baumgarten *et al.*, 2019; Barcons-Simon *et al.*, 2020). Baumgarten *et al.* targeted the putative m⁶A methyltransferase PfMT-A70 for knockdown after a knockout approach with the canonical CRISPR-Cas system was not successful. They designed a sgRNA complementary to a region downstream of the gene's TSS as well as ca. 100 bp upstream of the translation start site. A non-specific sgRNA served as control. They synchronized parasites and harvested samples 12, 36 and 48 hours post invasion (hpi). RT-qPCR analysis showed a significant downregulation of PfMT-A70 transcripts of 15% at 12 hpi and 40% at 36 and 48 hpi compared to samples from the non-specific control sgRNA line. The rationale for using CRISPRi in the other study (Barcons-Simon *et al.*, 2020) was similar since a simultaneous knockout of all 15 highly homologous members of a GC-rich non-coding RNA family was unsuccessful. Here, they cleverly designed a sgRNA that targeted a region that was common to all members in addition to a scrambled sgRNA as control. As in the other studies, the researchers could show dCas9 enrichment at the target loci with ChIP-seq and ChIP-qPCR. Using RT-qPCR, they found a significant reduction in non-coding RNA transcript levels in the knockdown strain compared to the strain that had received the control sgRNA.

In 2022, the group that published the system comprising dCas9 with its effector domains in *P. falciparum* (Xiao *et al.*, 2019) managed to make this system inducible by means of the DiCre/*loxP* system (Liang *et al.*, 2022) (Fig. 1.11 C). The authors reasoned that constitutive gene activation or repression by the CRISPRi machinery may lead to adaptation of the parasites and mask the true phenotype. Additionally, in case of targeting essential genes, the constitutive interference may be too harmful for parasites to allow a meaningful readout (Liang *et al.*, 2022).

At first, they had tried a system called TetR-DOZI for inducibility but were faced with leaky expression. For the DiCre/*loxP* system, they designed a plasmid where they inserted a GFP reporter construct flanked by two *loxP* sites upstream of the dCas9 expression cassette (Fig. 1.11 C) and transfected this plasmid into parasites that constitutively expressed DiCre. Accordingly, in the absence of rapamycin the parasites expressed GFP, whereas in the presence of rapamycin the reporter cassette was excised and the dCas9 cassette was expressed. They checked the efficiency and specificity of induction and found that the system provided robust and leak-free inducibility. With the help of this system, they targeted 8 genes and used 2-3 sgRNAs per gene. They found that, generally, sgRNAs targeting regions closest to the major TSS produced the most potent activation or repression, but the local chromatin status also seemed to have played a role. Like the study by Walker and Lindner (Walker and Lindner, 2019), they found that binding of a sgRNA in a known transcription factor binding region increased the regulatory effect (in the case of Walker and Lindner repression and in the case of Liang *et al.* activation of the respective target gene).

Interestingly, the same group recently published a paper where they adapted this experimental setup to perform screens (Lucky *et al.*, 2024). For this, they created pools of cell lines that they had used in the Liang *et al.* (2022) paper in addition to a cell line targeting a new gene. Therefore, these cell lines had the DiCre/*loxP*-inducible dCas9-GCN5 or dCas9-Sir2a background and had been transfected with a plasmid for expression of a sgRNA targeting one of these nine genes. The first screening setup consisted of just competitive growth behavior between the cell lines for which the researchers mixed equal numbers of synchronized parasites of each line having the dCas9-GCN5 or dCas9-Sir2a backgrounds, respectively, and subsequently induced dCas9-GCN5 or dCas9-Sir2a expression. Non-induced cells served as control. They used the same experimental approach to examine if an up- or downregulation of the targeted genes led to a difference in parasite fitness if the parasite pools were challenged with the drugs chloroquine or dihydroartemisinin, or with starvation or heat shock conditions, respectively. Since their overall rationale and some of the experimental strategies are quite similar to what I intended and performed in a small-scale screen in this thesis (see chapter 2.5.1), I will discuss their screening approach and its results in more detail in the Discussion (see chapter 3.2).

Researchers have declared in several of the studies mentioned in this chapter that they used CRISPRi because a knockout approach via canonical CRISPR-Cas was not successful or as an extension of the methodological toolbox in this parasite because it allows targeting of essential genes (Baumgarten *et al.*, 2019; Walker and Lindner, 2019; Barcons-Simon *et al.*, 2020). On the one hand, this highlights the complementary role CRISPRi plays to canonical CRISPR-Cas in

respect to individual targets. On the other hand, it alludes to an important fact: *Plasmodium* parasites are haploid in their asexual blood-stage form, so knockout approaches targeting essential genes will, by definition, not yield viable parasites. This is crucial to consider when designing screens that are supposed to fulfill the much needed and anticipated purpose of elucidating the function of ca. 75% of the parasite's genome that, so far, have only a putative or no known function at all (Böhme *et al.*, 2019). While not all these genes are essential, essential genes are often targets for therapeutic intervention or vaccine candidates (Ali *et al.*, 2021; Kimmel *et al.*, 2023), so that employing an approach that does not disregard this class of genes is highly desirable.

All studies mentioned here have employed only a relatively small number of sgRNAs per target gene. The study by Walker and Lindner constituted an exception where one sequence was targeted by 11 sgRNAs but even there the biggest effect in efficiency was shown by sgRNAs that happened to bind in a binding motif for the transcription factor PfAP2-G (Walker and Lindner, 2019). Therefore, it is so far not possible to deduce any comprehensive and reliable rules for designing efficient sgRNAs for CRISPRi in *Plasmodium*. In addition, the studies that used dCas9 fusions with effector domains must be viewed separately in this regard since both histone deacetylases and histone acetyltransferases can have an influence on the epigenetic landscape across larger ranges than dCas9 protein on its own (Walker and Lindner, 2019).

1.7 Thesis objectives

As mentioned before, the functional annotation of the genome of the deadliest human malaria parasite, *P. falciparum*, has progressed slowly. Accordingly, as of today, 32.7% of genes have no function assigned to them and 40.6% have only a putative function assigned. However, having a good understanding of the functions of the parasite's genes sets a crucial foundation for a multitude of ensuing research endeavors, both into the exciting divergent biology of *Plasmodium* but also in a more applicable/applied sense towards highly anticipated new avenues for therapeutic intervention and vaccine goals. So far, large-scale approaches targeting the whole genome of *P. falciparum* (Zhang *et al.*, 2018) and ca. half of *P. berghei*'s genome (Bushell *et al.*, 2017), relied on gene inactivation by different knockout methods that unfortunately do not, by themselves, permit functional exploration of essential genes.

We therefore envisioned that a knockdown approach, where the expression of the target gene is not entirely abrogated, would open the possibility for a measurable phenotype of targeted essential genes. Knockdown by CRISPRi, which has been established in *P. falciparum*, offers an

exquisite framework for this endeavor since, here, the target specificity is conferred by the 20 nt spacer sequence that can be introduced into library constructs in bulk (Joung *et al.*, 2017), which makes the approach easily scalable. Since the PAM of dCas9, which is most often used in CRISPRi, is -NGG, *P. knowlesi* was used for this project because its genome has a more balanced GC-content (37.5% (Pain *et al.*, 2008)) compared to that of *P. falciparum*. Although *P. knowlesi* is not as closely related to *P. falciparum* as to *P. vivax*, it is besides *P. falciparum* the only *Plasmodium* strain that has been adapted to continuous culture in human RBCs. In addition, we reasoned that the fact that ca. 80% of *P. knowlesi*'s predicted genes have orthologs in *P. falciparum* (Pain *et al.*, 2008), a number that increases to >90% if clonally variant gene families are disregarded, provided enough similarity between these two strains.

Accordingly, in this thesis project, I aimed to:

- I) Optimize transfection efficiency for sufficient sgRNA library representation
- II) Establish and validate CRISPRi technology in *P. knowlesi*
- III) Select a pool of high quality sgRNA
- IV) Establish sgRNA identification in a small-scale dropout screen by RT-qPCR

For II), we sought to validate knockdown via CRISPRi with both essential and non-essential genes. The selection was based on a combination of thematic interest and practical reasons, the latter of which I will further elaborate in the Results chapter concerned with knockdown validation (see chapter 2.4.2).

1.7.1 Target gene selection

Given the interest our group has in the intricate mechanisms of the parasite's asexual replication, analyzing a knockdown phenotype of centrin genes seemed quite tempting. In general, centrins are small structural proteins that localize to the centrosome which is the microtubule organizing center (MTOC) for the mitotic spindle. The MTOC of *Plasmodium* is called the centriolar plaque and seems to be organized as a bipartite structure that spans the nuclear envelope in *P. falciparum* (Fig. 1.12 A) (Simon *et al.*, 2021). Recently, Voß *et al.* have shown that all 4 centrin proteins (Cen1-Cen4) of *P. falciparum* localize to the centriolar plaque in this strain (Voß *et al.*, 2023) (Fig. 1.12 B). The exact function of centrins is still elusive but implicated functional roles range from *de novo* assembly of centrioles in humans (Yang *et al.*, 2010) to the duplication of the spindle pole body which is the MTOC in yeast (Rüthnick *et al.*, 2021). In *Toxoplasma gondii*, another *Apicomplexan* parasite, the depletion of one centrin protein (TgCen2) led to aberrant replication (Leung *et al.*, 2019). A study from 2019 which investigated the phenotype/function of centrins

in *P. berghei* found that Cen1-Cen3 were refractory to knockout as well as tagging at their N- or C-terminus (Roques *et al.*, 2019). This was in line with the results of a large-scale screen in *P. berghei* from 2017 where *PbCen1* and *PbCen2* were categorized as essential (no data on *PbCen3* or *PbCen4*) (Bushell *et al.*, 2017). Interestingly, Roques *et al.* were able to C-terminally tag *PbCen4*, one of two *Alveolata*-specific centrin proteins in *Plasmodium*, with GFP and thereby follow its cellular localization during multiple stages of the parasite's life cycle (Roques *et al.*, 2019). Even more surprising, *PbCen4* could be knocked out with no significant effect on parasite growth or phenotype throughout all life cycle stages, suggesting the gene's dispensability (Roques *et al.*, 2019). Although there seem to be conflicting results regarding the dispensability of *PbCen4* the possibility of targeting a reportedly dispensable centrin protein alongside a reportedly essential one represented a great opportunity for the development of this thesis. Especially so since efforts in our group to genetically modify genomic *PfCen1* and *PfCen3* have been unsuccessful (Voß *et al.*, 2023), as was the case for *PbCen1*-Cen3 (Roques *et al.*, 2019).

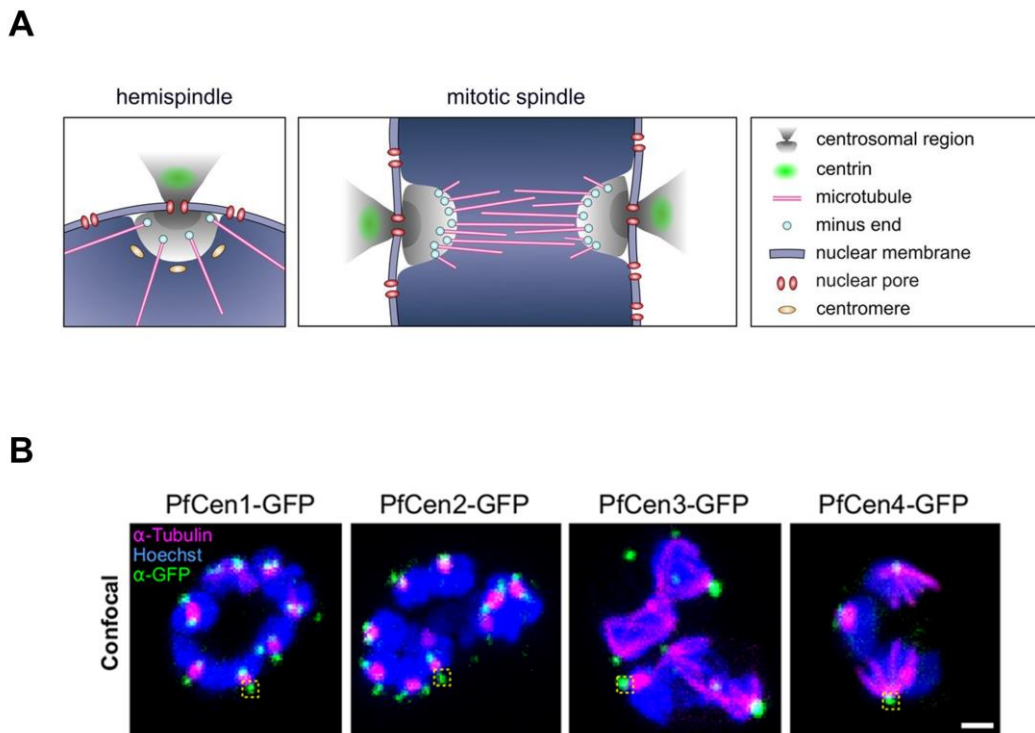


Fig. 1.12: Centrin localization in *P. falciparum*. (A) The centriolar plaque in *P. falciparum* is a bipartite structure spanning the nuclear membrane. Centrin is localized in the outer core. This panel was taken from (Simon *et al.*, 2021). (B) In *P. falciparum*, all 4 centrin proteins have been shown to localize to the centriolar plaque. This panel was modified from (Voß *et al.*, 2023).

As the second non-essential gene we chose SAS6 that codes for the spindle abnormal protein 6 (Sas6) which is also associated with the centrosome. In organisms that have centrioles, Sas6 localizes to the inner core of the characteristic cylindrical shape of these structures (Fig. 1.13). The protein consists of a globular portion that homodimerizes and assembles into a circular array as well as long coiled-coil domains that extend from this central hub like spokes of a cartwheel (Fig. 1.13) (Kitagawa *et al.*, 2011). At the outer part of the spokes 9 triplets of microtubules assemble. Intriguingly, centrioles share the same basic structure as the basal body, but they can have quite diverse functions, depending on the context: ranging from forming cilia and flagella to the already mentioned centrosome assembly (Carvalho-Santos *et al.*, 2010; Pearson, 2014). In the asexual blood-stage forms of *P. falciparum*, neither centrioles nor basal bodies have been detected at present and the centriolar plaque appears to be amorphous (Simon *et al.*, 2021) but a homolog of SAS6 is present in the *Plasmodium* genome. So far, it had mainly been implicated in the basal body and flagellum assembly in male gametocytes (Marques *et al.*, 2015). In line with this, *PfSas6* has been deemed dispensable for asexual blood-stage growth (Zhang *et al.*, 2018). It has even been suggested that Sas6 is absent in asexual blood-stages, so that their expression is stage-specific for sexual forms (Wall *et al.*, 2016). However, a large-scale transcriptome analysis of *P. falciparum* during the IDC showed at least transcription of the SAS6 gene, though not to a very high level (Otto *et al.*, 2010).

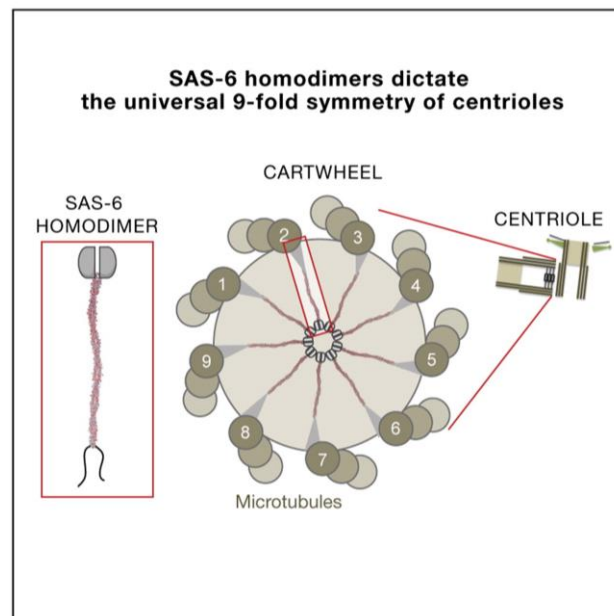


Fig. 1.13: SAS-6 localizes to the inner core of centrioles. SAS-6 proteins form homodimers and their globular portions arrange in a circle so that the filamentous coiled-coil portions protrude outward like spokes of a cartwheel. At the end of these spokes, triplets of microtubules bind, giving the centrioles their characteristic 9-fold symmetrical appearance.

The final gene we chose to target is the vacuolar protein sorting associated protein 32 (VPS32; also called SNF7). It is part of the endosomal sorting complex required for transport (ESCRT)-III, that is involved in vesicle formation in general, and various other processes, including cytokinesis, nuclear envelope remodeling and exosome biogenesis (Hurley, 2015). The ESCRT usually consists of four complexes, ESCRT-0, -I, -II and -III, and ESCRT function relies on the sequential recruitment and activation of these complexes (Avalos-Padilla *et al.*, 2021). Curiously, intracellular protists, such as *Plasmodium* spp., appear to encode only some members of ESCRT-III and none of the other complexes in their genome which led to the hypothesis that an alternative pathway of activation of ESCRT-III might exist in *Plasmodium* (Avalos-Padilla *et al.*, 2021). Indeed, Avalos-Padilla *et al.* identified a protein they called *Pf*BroI that, if added in reconstituted form to giant unilamellar vesicles in the presence of reconstituted *Pf*Vps32, was able to activate membrane bud formation (Avalos-Padilla *et al.*, 2021). Intriguingly, they were unable to generate knockouts of both *Pf*Vps32 and *Pf*BroI which they attributed to a potential essential role in the asexual blood-stage, which is supported by its previously reported essential phenotype in the same strain (Zhang *et al.*, 2018).

2. Results

2.1 Generation of marker-free parasite line with stable dCas9 expression

With the goal of establishing CRISPRi in *P. knowlesi* in mind, Ann-Kathrin Mehnert generated a cell line with constitutive dCas9 expression prior to the beginning of this project (A.-K. Mehnert, Master thesis, 2019). For clarity, I will reiterate her approach here. In principle it is possible to have constitutive expression from an episomally maintained plasmid. However, since the number of available resistance cassettes for selection of parasites that carry the respective plasmid after transfection (and further maintenance of this plasmid) are limited in *Plasmodium* parasites (Balu and Adams, 2007), integration of the dCas9 expression cassette was favored. For this, the *p230p* locus was chosen since it had been shown to be dispensable in *P. berghei* (Janse *et al.*, 2006) and had been used for transgene integration in *P. knowlesi* before (Moon *et al.*, 2013; Mohring *et al.*, 2019).

The integration was achieved via CRISPR-Cas9 with a two-plasmid strategy (Fig. 2.1 A). The pCas9/sg_p230p plasmid, that was kindly provided by Robert Moon (Mohring *et al.*, 2019), supplied the Cas9 and a specific sgRNA targeting the *p230p* locus for introducing the DSB. This plasmid contains a fused human dihydrofolate reductase/yeast cytosine deaminase and uridyl-phosphoribosyltransferase (hDHFR/yFCU) cassette that enables positive selection with WR99210 and negative selection with 5-fluorocytosine, respectively. Ann-Kathrin Mehnert amplified the dCas9 sequence tagged with a triple-HA-tag and a *PjHSP70* 5' UTR as well as a *PbDT* 3'UTR from a plasmid kindly provided by Artur Scherf (Baumgarten *et al.*, 2019). Afterwards, she cloned this expression cassette flanked by 400 bp homology arms on each side into the pDonor_p230p plasmid, which was also provided by Robert Moon (Mohring *et al.*, 2019), thereby building the pPk-dCas9-p230p vector. The amplified dCas9 cassette included a glucosamine-6-phosphate activated (*glmS*) ribozyme sequence upstream of the 3' UTR which provided the option of dCas9 expression downregulation if it became necessary or desired.

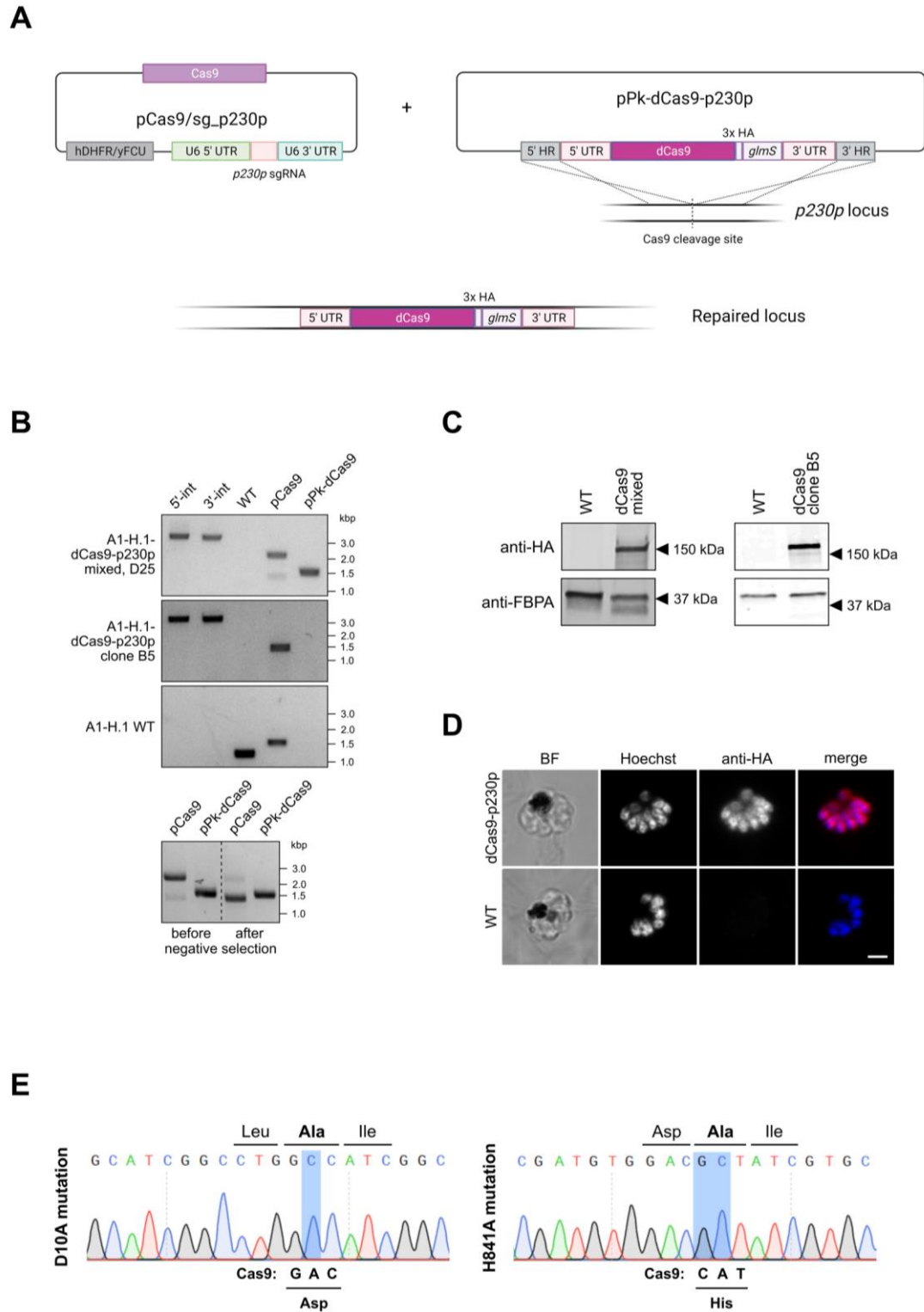


Fig. 2.1: Generation of a marker-free *P. knowlesi* line with stable dCas9 expression. (A) Plasmid strategy for integrating a dCas9 expression cassette into the *p230p* locus of the *P. knowlesi* A1-H.1 WT strain using CRISPR-Cas9. The pCas9/sg_p230p plasmid (left) provided the sequence for Cas9 expression, a cassette for expression of sgRNA for guiding the Cas9 to the *p230p* locus and a human dihydrofolate reductase /yeast cytosine deaminase and uridyl-phosphoribosyltransferase (hDHFR/yFCU) cassette for positive/negative selection of the plasmid, respectively. The pPk-dCas9-p230p plasmid (right) carried the dCas9 expression cassette (dCas9-3xHA-*glmS*) to be integrated, flanked by 5' and 3' homology regions for facilitating homology-directed repair. This panel was created with BioRender.com. (B) Agarose gels of diagnostic PCRs that checked for (top panel) correct 5' integration (5'

int), correct 3' integration (3' int), WT locus (WT), pCas9/sg_p230p plasmid (pCas9), pPk-dCas9-p230p plasmid (pPk-dCas9) in the non-clonal transfected line (A1-H.1-dCas9-p230p mixed, D25 (refers to day after transfection when sample was taken)), the clonal line B5 (A1-H.1-dCas9-p230p clone B5) and the WT (A1-H.1 WT). The gel on the bottom shows presence of pCas9/sg_p230p plasmid (pCas9) and pPk-dCas9-p230p plasmid (pPk-dCas9) before and after negative selection with 5-fluorocytosine. **(C)** Western blot analysis of expression of dCas9-3xHA with anti-HA antibodies in the non-clonal line (dCas9 mixed) and the clonal line B5. Fructose-bisphosphate aldolase (FBPA) served as loading control. **(D)** Immunofluorescence analysis of dCas9-3xHA expression and localization. BF: brightfield. Blue channel: Hoechst, red channel: anti-HA. Scale bar 2 μ m. **(E)** Chromatogram showing Sanger-sequenced samples of dCas9 clone B5 parasites. The blue shading indicates presence of D10A (left) and H841A (right) mutations while the original sequence of Cas9 is indicated below. Figures were adapted from (A.-K. Mehnert, MSc thesis, 2019).

Ann-Kathrin Mehnert then co-transfected the two plasmids into *P. knowlesi* A1-H.1 WT parasites which reappeared (following clearance by WR99210) 12 days after transfection. She extracted genomic DNA (gDNA) from parasite samples taken 13 and 25 days after transfection, respectively, and tested it for the presence of WT locus and correct integration of the dCas9 expression cassette by diagnostic PCRs (Fig. 2.1 B). While the WT locus was still detectable 13 days after transfection, it had disappeared by day 25 after transfection (Fig. 2.1 B). After she ensured correct 5' and 3' integration, she removed WR99210 for 7 days to allow the parasites to lose the pCas9/sg_p230p plasmid, before she started negative selection of the plasmid. Finally, she generated the clonal lines *P. knowlesi* A1-H.1-dCas9-B5, -D7 and -F6, respectively, by limiting dilution cloning with a plaque-based approach (Thomas *et al.*, 2016; Mohring *et al.*, 2019). For all three clones neither the WT locus, nor the two plasmids used for generation of this line were detectable by PCR (Fig. 2.1 B).

Ann-Kathrin Mehnert confirmed the expression of the dCas9 in the non-clonal A1-H.1-dCas9-p230p line by western blotting using anti-HA antibodies where she was able to detect the corresponding band at the expected size of ca. 150 kDa (Fig. 2.1 C). Using immunofluorescence assay (IFA), with anti-HA antibodies as well, she further observed that the dCas9 localization was enriched in the nucleus of these parasites which was expected (Fig. 2.1 D). I later analyzed dCas9 expression by IFA in the A1-H.1-dCas9-B5, -D7 and -F6 clones (hereafter, referred to as e.g., "dCas9 B5") which confirmed the presence of the HA-tagged dCas9 in these clones (Suppl. Fig. 1). It also showed that its localization seemed to be enriched in the nucleus to varying degrees.

To verify that the dCas9 contained the point mutations necessary to render it catalytically inactive, Ann-Kathrin Mehnert had the PCR-amplified sequences used for diagnostic PCRs of the *Pk*A1-H.1-dCas9-B5 clone Sanger-sequenced which confirmed the D10A and H841A mutations (Fig. 2.1 E).

2.2 Assessment of transfection efficiency

The transfection efficiency is an important parameter for a screening process because it determines the size limit of the library, i.e., the number of constructs that can be transfected while still achieving its complete representation in the cell population. To reliably ensure complete representation of the constructs in CRISPR-Cas9-based screens, it is recommended to use a library at a coverage of 500-1000x, meaning 500-1000 cells transfected per sgRNA (Joung *et al.*, 2017; Bock *et al.*, 2022). CRISPR-Cas9 systems using mammalian cells have been used extensively so that it was possible to determine prerequisites for designing highly efficient sgRNAs (Doench *et al.*, 2014, 2016; Gilbert *et al.*, 2014; Labuhn *et al.*, 2018). In the beginning of this process, studies have used 10 sgRNAs per gene target to ensure efficient disruption of gene expression (Gilbert *et al.*, 2014; Wang *et al.*, 2014) which has since been reduced over time to ca. 5 sgRNAs per gene target (Joung *et al.*, 2017). In my case, using a parasite for which these sgRNAs requirements are almost completely unknown, I would deem it necessary to use 10 sgRNAs per gene target so that efficient gene disruption is ensured, and design rules can be deduced from the screen's results.

Accordingly, as an example, if one wanted to transfect a genome-wide library for *P. knowlesi* (A1-H.1 strain), the following parameters could be envisioned:

- (I) Adhering to the above-mentioned parameter of achieving **500 cells** transfected per sgRNA
- (II) Designing **10** sgRNA **constructs** per target gene (**4950** protein-coding **genes**)
- (III) Assuming a **10%** survival during electroporation
- (IV) Assuming a **10%** efficiency of transfection

From this the following equation ensues:

$$No.parasite\ cells = 10\ constructs \times 4950\ genes \times 500 \frac{cells}{construct} \times 10\% survival \times 10\% efficiency$$

$$No.parasite\ cells = 2.5 \times 10^9$$

Therefore, with the example parameters and assumptions given above, one would need to use 2.5×10^9 parasites during transfection to ensure complete representation of a genome-wide library in *P. knowlesi*. This corresponds to either 500 μ l purified schizont-stage parasites or 10 ml of iRBCs at a parasitemia of 5%.

2.2.1 Strategy 1: Transfecting GFP-expressing construct and measuring GFP-positive population with flow cytometry

To test the transfection efficiency of the dCas9 strain, I first transfected a reporter construct that expressed enhanced green fluorescent protein (eGFP; hereafter “GFP”) and measured the GFP-positive (GFP(+)) fraction of the parasite population at different time points after transfection. In the reporter construct pPkconGFP, which was kindly provided by Robert Moon at LSHTM in London (Moon *et al.*, 2013), the expression of GFP is controlled from a *Pk*HSP70 5' UTR and a *Pb*DT 3' UTR (Fig. 2.2 A). Selection for this construct happens via expression of human dihydrofolate reductase (hDHFR) from a *Pk*EF1a 5' UTR and a *Pf*HRP2 3' UTR that confers resistance against the drug WR99210.

Although Moon *et al.* originally assessed the transfection efficiency using this reporter construct by counting GFP(+) parasites from microscopy images (Moon *et al.*, 2013) we decided to quantify the GFP(+) fraction of parasites by flow cytometry because it seemed to be the experimental option that gives more accurate results while being less laborious, especially when several samples were measured.

In fact, I planned to perform experiments in which I transfected both GFP- and mScarlet-expressing constructs (separately and pooled) to not only assess the transfection efficiency but also the multiplicity of vector uptake which also plays an important role for a meaningful readout of a screen (Suppl. Fig. 2). For this, I exchanged the GFP CDS in pPkconGFP with the mScarlet CDS (Fig. 2.2 B).

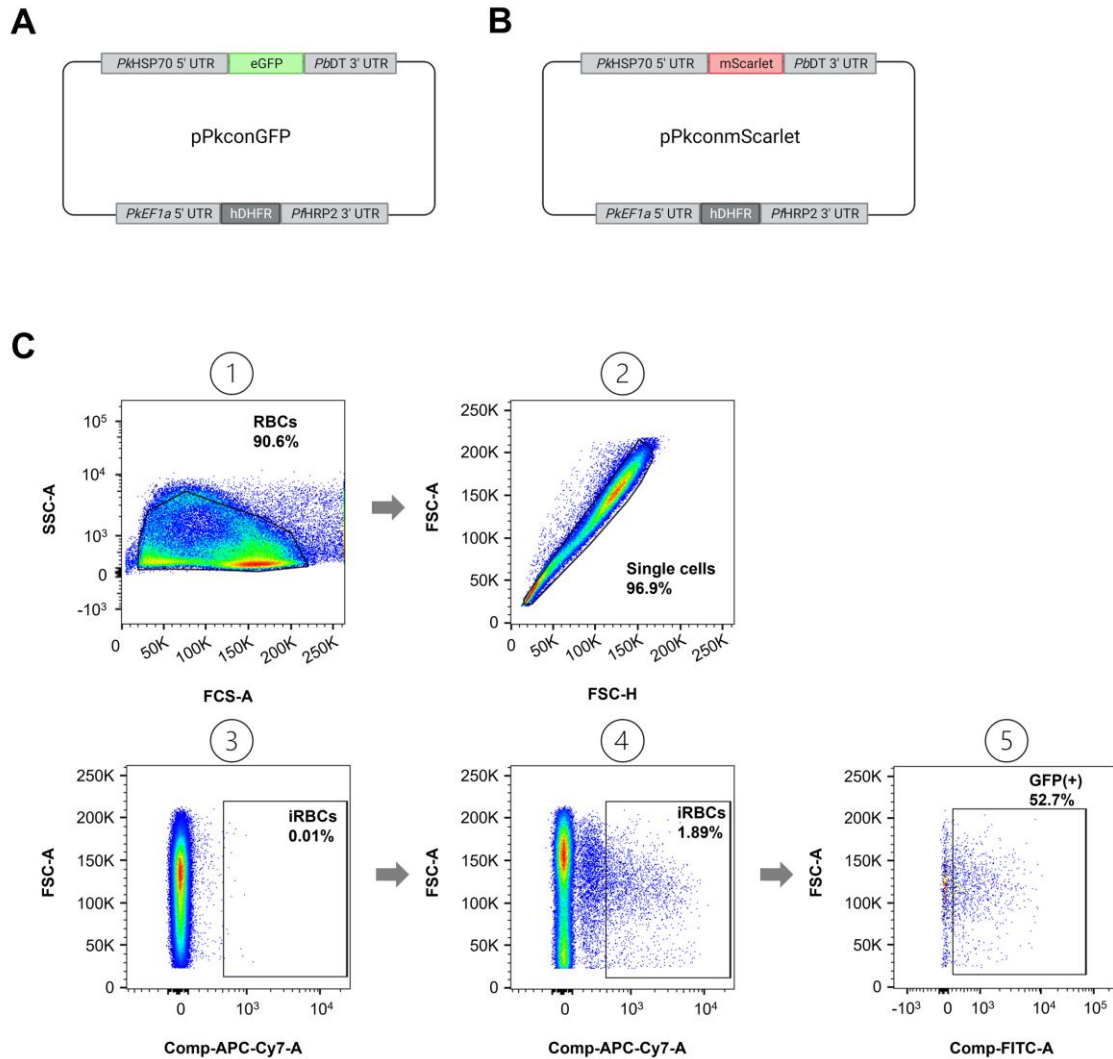


Fig. 2.2: Assessing the transfection efficiency with reporter constructs revealed a large proportion of parasites not expressing GFP despite selection pressure. (A) Reporter construct driving GFP expression from *P. knowlesi*-specific promoter (PkHSP70 5' UTR). The plasmid includes a human dihydrofolate reductase (hDHFR) cassette for positive selection using the drug WR99210. (B) Reporter construct was made by exchanging the GFP with an mScarlet CDS. (A) and (B) were created with BioRender.com. (C) Gating strategy for measuring the GFP(+) parasite population after 13 days of continuous positive selection. First, the bulk of RBCs was selected (1), then the applied gating selected single cells (2). Using an unstained control sample the gating was set for infected RBCs (iRBCs) (3), the percentage of which was then measured using the sample stained for DNA (4). Finally, using a non-fluorescent sample (not shown) the gating was set to capture GFP(+), the percentage of which can be seen in (5). In this case, only 52.7% of parasites expressed the reporter GFP after 13 days of continuous selection pressure.

To assess the efficiency of transfection, I performed four independent transfections with the dCas9 B5 strain, comprising 1-6 reactions each. Since parasite survival and transfection efficiency remained low even after several optimization measures, I also performed two transfections with WT parasites to compare transfection efficiency and growth behavior of cultures after transfection. Selected parameters and observational results are summarized in Tab. 2.1.

In general, there were two main challenges to my experimental setup and readout, which persisted despite optimization measures that will be discussed in the following paragraph. These were (i) very low parasite survival during nucleofection (mostly ca. 0.001-0.005% with one exception of 1.7%) and (ii) the fact that there was a large fraction of the parasite population that remained GFP(-) even after prolonged culture under selection pressure. An example of this is shown in Figure 2.2 C, where after 13 cycles of growth under selection pressure, almost half of the drug-resistant population (43.8%) showed no detectable expression of GFP by flow cytometry. Therefore, using the GFP(+) fraction of the population would very likely be a gross underestimation of transfection efficiency.

Although it became clear that using the GFP(+) fraction was not a good approximation for the transfection efficiency, I still sought to analyze the optimization measures already employed to determine if they had a beneficial effect on transfection efficiency. For this, I relied on two different growth characteristics of the parasite cultures as indirect measures for transfection efficiency or a measure for transfection outcome/success since only successfully transfected parasites can grow under selection drug pressure. The first characteristic was the number of cycles it took the culture to reach 0.5% parasitemia and the second was the number of cycles it took the culture to enter exponential growth, i.e. until at least two consecutive cycles showed a ≥ 2 -fold increase in parasitemia. I chose these measures because they compensate each other well regarding the two potential bottlenecks of transfection efficiency: if the survival in a transfection experiment was low, the initial parasitemia would also be low (it might take more cycles to reach 0.5% parasitemia). Nevertheless, a relatively high percentage of the parasites that survived could still have received a plasmid, meaning that the culture will be able to grow well even under selection pressure (it might take less time to reach two consecutive cycles with ≥ 2 -fold increase in parasitemia).

Tab. 2.1: Selected parameters of transfections using reporter constructs to assess transfection efficiency. Over the course of 6 transfections using both the dCas9 B5 and the WT strain, several optimization measures were tested to increase transfection efficiency, e.g. the use of the egress inhibitor ML10 and/or different nucleofactors (4D and 2b). The number of parasites used during transfection was also recorded to assess if this influenced transfection efficiency. Two parameters were chosen to reflect transfection outcome/success from these data: the number of cycles it took until the transfected culture reached 0.5% parasitemia and the number of cycles it took until exponential growth of the culture was detected, defined as at least two consecutive cycles showing a ≥ 2 -fold increase in parasitemia. Numbers in *Italics* were calculated from smears (instead of counted).

* Parasitemia never went below 0.6%

Transfection	Strain transfected	Transfection reactions	Number of segmenters transfected	First parasitemia 0.5% [cycle]	time Exponential growth detected [cycle]
1	dCas9 (clone B5)	GFP	<i>1,9E+08</i>	9	9
		mScarlet	<i>1,9E+08</i>	9	9
		GFP/mScarlet	<i>1,9E+08</i>	9	11
2	dCas9 (clone B5)	GFP/mScarlet	<i>1,0E+08</i>	9	11
3	dCas9 (clone B5)	GFP	4,9E+07	11	12
		mScarlet	4,9E+07	8	11
		GFP/mScarlet	4,9E+07	8	8
		GFP + ML10	4,9E+07	6	12
		mScarlet + ML10	4,9E+07	5	8
		GFP/mScarlet + ML10	4,9E+07	6	12
4	dCas9 (clone B5)	GFP + ML10 (4D)	3,7E+07	8	NA
		GFP + ML10 (2b)	3,7E+07	6	6
5	WT	GFP+ML10 (2b)	2,5E+07	1*	NA
6	WT	GFP+ML10 (4D)	1,8E+08	2	3
	WT	GFP+ML10 (2b)	1,8E+08	2	3

One factor of the nucleofection process I sought to examine was the **number of segmented schizonts** used per transfection reaction. As reported in (Mohring *et al.*, 2020), the efficiency of transfection decreases disproportionally when less than 10 μ l purified *P. knowlesi* late-stage schizonts are used, which corresponds to ca. 10^8 parasites. I therefore aimed at obtaining at least this number of segmented schizonts which was unfortunately not always possible due to unexpected growth behavior of the parasite strain (will be elaborated in chapter 3.3.). For analyzing the effect of parasite number on the transfection outcome, I divided the transfection reactions according to the criterion if $<10^8$ or $\geq 10^8$ parasites were used and, interestingly, found that there was no significant difference between these two groups (Fig. 2.3 A). One explanation for this outcome could be that the values in the lower category were still quite close to 10^8 parasites. It could also be because most of the values in the upper category are likely (slight) overestimations since they were not obtained by counting the parasites after harvesting right

before transfection with a counting chamber but were calculated from smears taken before harvest.

The second factor I analyzed was the influence of the **nucleofactor used**. Since parasite survival during nucleofection remained unexpectedly low (ca. 0.001-0.004%) for the first three transfections, I used a second nucleofactor (Amaxa 2b) in addition to the one that had been used so far (Amaxa 4D) to compare efficiency. Although there were only three transfection reactions where the Amaxa 2b nucleofactor was used, parasites transfected with this nucleofactor reached 0.5% parasitemia as well as the exponential growth phase significantly faster than parasites that were transfected with the Amaxa 4D nucleofactor (Fig. 2.3 B).

In addition, I reasoned that optimizing the synchronicity of the parasites before transfection would likely enhance efficiency. It is assumed that electroporation of segmented late-stage schizonts, at least in *P. berghei*, induces egress (van Dijk, Waters and Janse, 1995; van Dijk *et al.*, 1997; Waters *et al.*, 1997) by rupturing the already weakened schizont membrane (Waters *et al.*, 1997). Not only could this increase the number of available and viable cells that could take up plasmids severalfold (which should be roughly equal to the growth rate of the respective strain), but it would also eliminate several (two or three, depending on if the parasitophorous vacuole membrane is still intact) of the membranes the plasmid must cross to get into the parasite's nucleus. Yet, this process can only be effective if the merozoites are fully segmented and developmentally autonomous so that they are viable in the surrounding medium until they can invade RBCs.

Therefore, to increase synchronicity, I incubated the cultures with **ML10**, an inhibitor of the protein kinase G that is responsible for starting an enzymatic cascade finally leading to the egress of merozoites (Ressurreição *et al.*, 2020). Since this process is inhibited during the time the compound is present, the proportion of segmented parasites is increased. It has been reported that, given the parasites have not been incubated longer than 4 h with ML10, and after the compound is washed out, normal egress can occur and parasites can still efficiently re-invade RBCs (Ressurreição *et al.*, 2020). Accordingly, when I treated parasites with ML10 for 2 h before transfection, they gave rise to cultures that reached 0.5% parasitemia significantly faster than parasites that were not treated (Fig. 2.3 C). Interestingly, the difference in number of cycles it took until the parasites entered exponential growth was not significant between cultures that had been incubated with or without ML10 (Fig. 2.3 C). This could indicate that incubation with ML10 improves parasite survival, e.g. by increasing the number of sufficiently developed segmenters before electroporation, but it might not specifically increase the number of parasites

that receive a plasmid during electroporation. Nonetheless, ML10 incubation was subsequently routinely performed before transfection.

Finally, I analyzed the effect the **parental strain** had on the transfection efficiency by comparing the growth characteristics for the dCas9 B5 strain and the WT strain. Importantly, transfection efficiency was significantly higher when the WT strain was used (Fig. 2.3 D). While this was not an optimization measure, it still made clear that, unfortunately and for unknown reasons, transfecting the dCas9 strain remained much less efficient than transfecting the WT strain.

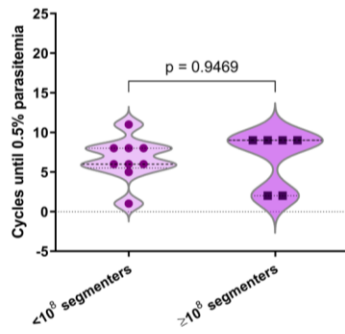
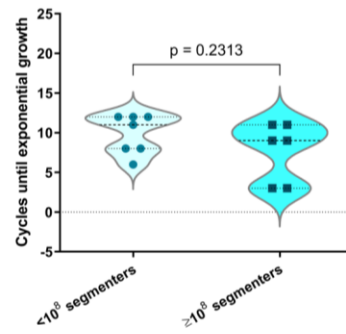
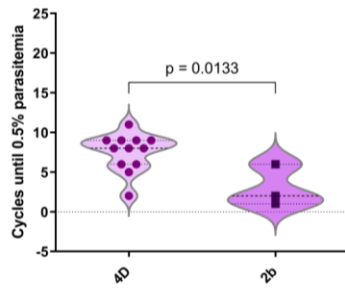
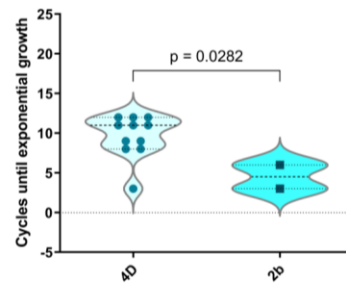
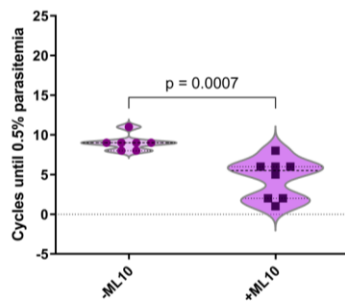
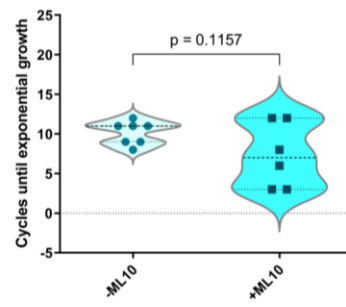
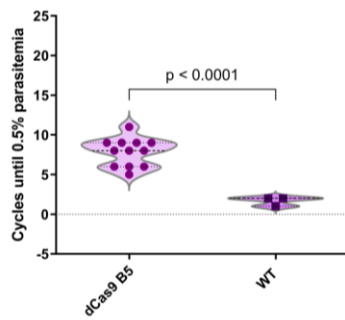
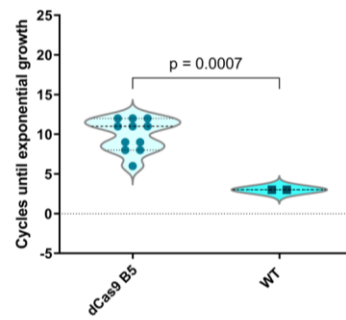
A**Effect of no. of parasites (parasitemia)****Effect of number of parasites (growth)****B****Effect of nucleofector (parasitemia)****Effect of nucleofector (growth)****C****Effect of ML10 (parasitemia)****Effect of ML10 (growth)****D****Effect of strain (parasitemia)****Effect of strain (growth)**

Fig. 2.3: Analyzing the effect experimental parameters had on transfection success/outcome. Violin plots visualizing the effect the number of parasites used in transfection reactions (A), nucleofector used for transfection (B), use of egress inhibitor ML10 (C) and strain transfected (D) had on transfection success/outcome. The transfection success was determined by identifying the number of cycles it took the respective cultures to reach 0.5% parasitemia ((A)-(D) “parasitemia”, left) and the number of cycles it took the culture to reach exponential growth ((A)-(D) “growth”, right) after transfection. Statistical significance was determined by unpaired, two-tailed t-test using GraphPad Prism. *Note: the number of data points for “parasitemia” and “growth” differs since two data points were missing from the “growth” observation. See also Tab. 2.1 for this.*

2.2.2 Strategy 2: Transfecting sgRNA construct and using resistance against selection drug WR99210 as readout

As mentioned in the previous subchapter, when I measured the GFP(+) fraction of parasites after transfecting the reporter, roughly half of the parasites did not express the fluorescent reporter, even after nearly 2 weeks under continuous drug pressure (Fig. 2.2 C (5)). Therefore, we were not confident that this experimental setup would yield accurate transfection efficiency results. Instead, I decided to use resistance against the selection drug WR99210 as a readout for transfection efficiency since growth under selection pressure is the minimal requirement for successful transfection and it presented two crucial advantages. Firstly, it allowed me to examine the parasite survival during the transfection reaction independently from the overall transfection efficiency which will be elaborated in the following paragraph. Secondly, I was able to transfect sgRNA constructs to generate the first knockdown cultures (instead of reporter constructs) that could subsequently be used for knockdown validation (see chapter 2.4.2).

As established previously, it is assumed that transfecting late-stage schizonts releases merozoites during the process of electroporation (van Dijk, Waters and Janse, 1995; Waters *et al.*, 1997). Thus, to determine the parasites’ survival of nucleofection, I first calculated the number of merozoites that are theoretically released during this process by counting the number of segmented schizonts before transfection in a Neubauer chamber and multiplying this number by the growth rate (R) the strain exhibited in the days before transfection. It should be noted that I hereby assumed the egress efficacy to be 100%:

$$No. merozoites = No. segmented schizonts \times R$$

The number of parasites capable of RBC invasion after a recovery period of 30 min (see chapter 4.2.1.6) were counted and used as a proxy for parasite survival. The survival is, hence, the number of parasites that invaded RBCs (ring-stage parasites) divided by the number of merozoite-stage parasites that were theoretically available during nucleofection:

$$Parasite survival [\%] = \left(\frac{No. parasites after transfection [ring - stage]}{No. parasites before transfection [merozoites]} \right) \times 100$$

Since I had not been able to determine the parasites' egress efficacy during electroporation, it is relatively likely that the parasite survival is underestimated to an unknown degree by this calculation.

To determine the transfection efficiency, separate from the parasites' survival, it was necessary to determine the number of cycles that had passed after nucleofection until all parasites not carrying a plasmid had died. Consequently, I included a mock transfection reaction in which I electroporated the parasites without adding plasmid DNA, along with the reactions that received sgRNA constructs (in this case targeting the CEN4 gene, see also chapter 2.4.2) (Fig. 2.4 A). Figure 2.4 B depicts a general schematic of the respective constructs. Before the selection drug WR99210 was added, I split the mock-transfected parasite culture in two halves: one received the usual amount of selection drug (Mock+WR), the other one was left without (Mock-WR) (Fig. 2.4 A). The Mock+WR condition was used to indicate the timepoint at which all parasites that did not receive plasmid DNA, by definition, have died and the Mock-WR condition was used to determine the normal growth rate of the electroporated strain without selection pressure. In two biological replicates of transfection, all parasites in the Mock+WR were detected to be dead in the third cycle after transfection (Fig. 2.4 C). With this information, the efficiency was calculated as follows:

$$\text{Transfection efficiency (\%)} = \left(\frac{N_p}{R^n} \right) \times 100$$

N_p is the number of parasites present in the cycle where all parasites in the Mock+WR condition had died, and R^n is the growth rate R (determined by the Mock-WR condition) to the power of number of cycles that had passed since nucleofection.

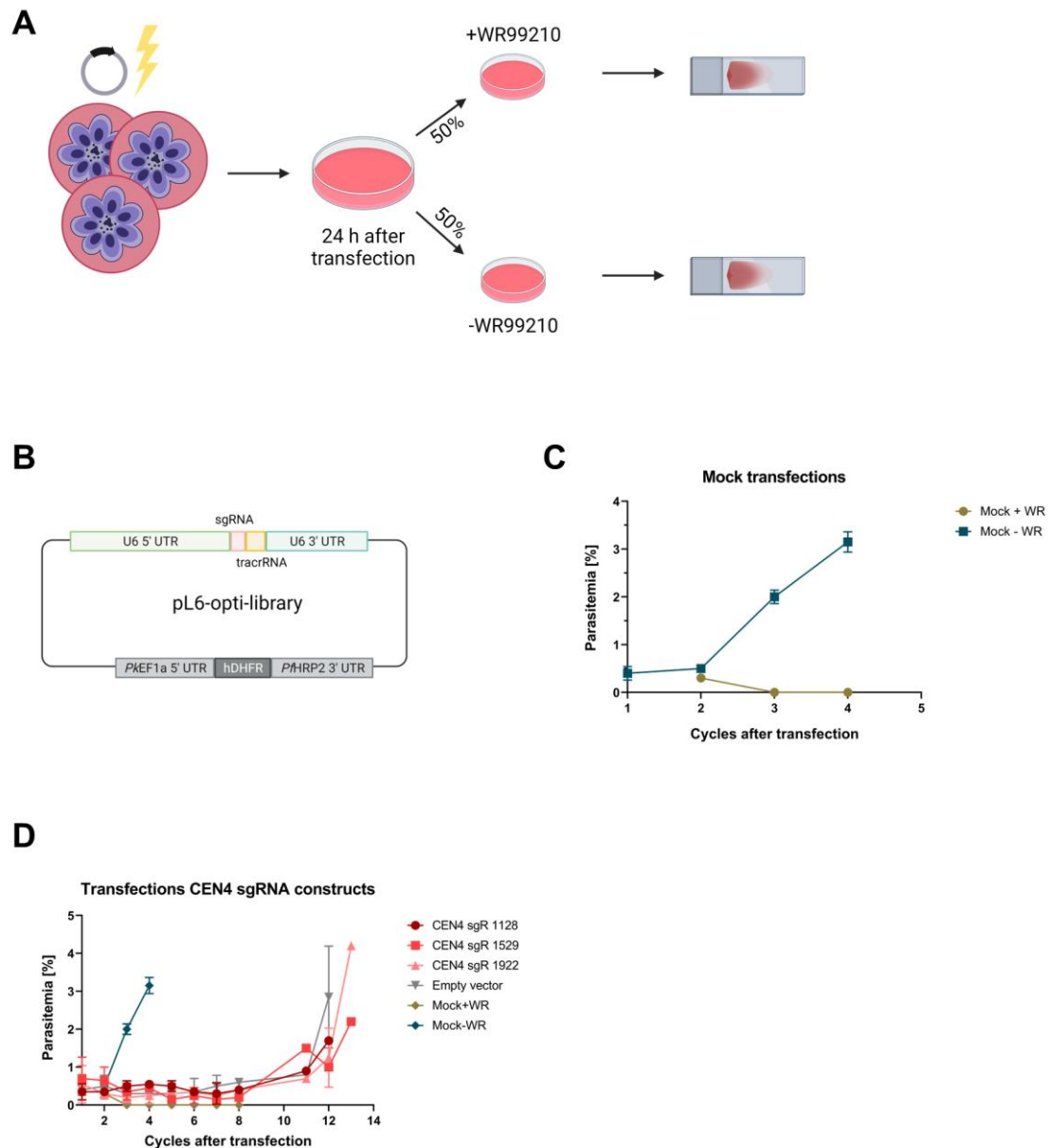


Fig. 2.4: Assessing the transfection efficiency via the parasites' resistance against WR99210. (A) Schematic visualizing experimental strategy used to determine number of cycles it took until parasites without plasmid containing selection cassette had died from selection drug (+WR99210) as well as normal parasite replication rate (-WR99210) after transfection in mock transfection reactions. Here, parasites were transfected without the addition of plasmid DNA. After 24 h, the culture was split and half received the normal amount of selection drug WR99210, while the other half received no drug. Thin blood smears were made from both cultures to monitor parasite growth. (B) Schematic of the plasmid structure of sgRNA constructs. Expression of the sgRNA, including the spacer (light red) and an optimized tracrRNA (yellow), is driven from U6 5' and 3' UTRs. The human dihydrofolate reductase (hDHFR) expression cassette for positive selection is the same as in the reporter constructs (Fig. 2.2 (A) and (B)). (A) and (B) were created with BioRender.com. (C) Growth curves of the mock-transfected parasite cultures in the presence (+WR, light brown) or absence (-WR, blue) of the selection drug from two biological replicates. (D) Growth curves of mock-transfected parasites (light brown and blue), as well as parasites transfected with 3 different sgRNAs against the CEN4 gene (CEN4 sgR 1128, CEN4 sgR 1529, CEN4 sgR 1922; different shades of red) and an empty vector control (grey). (C) and (D), each symbol is the mean from n=2 biological replicates, and the error bars are the standard deviation.

In the two replicates of transfections I have performed using these conditions, the mean survival was low, between $1.4 \pm 0.4\%$ and $2.7 \pm 0.4\%$ (average across sgRNA constructs $2.0 \pm 0.4\%$ (Tab. 2.2). The transfection efficiency of those parasites that survived nucleofection was between $10.0 \pm 0.1\%$ and $28.2 \pm 2.1\%$ (average across sgRNA constructs $16.3 \pm 7.6\%$. The overall efficiency was determined by multiplying these two factors which showed that the average overall efficiency was $0.3 \pm 0.1\%$ which is ca. 100-fold lower than reported by Moon *et al.* (Moon *et al.*, 2013).

Tab. 2.2: Parasite survival during transfection reaction and transfection efficiency as determined via parasite resistance against selection drug WR99210. The percentage of parasites surviving the transfection were determined by counting the number of segmented schizonts before the transfection and ring-stage parasites after the transfection and a recovery period. The efficiency was determined by calculating back from the timepoint where all the parasites that did not receive a plasmid had died (for both calculations, see formulas in main text). The overall transfection efficiency was then calculated by multiplying the survival and the efficiency. SD: standard deviation.

Condition	Survival (%) (mean $\pm$ SD)	Efficiency (%) (mean $\pm$ SD)	Overall efficiency (%)
CEN4 sg R1128	1.4 ± 0.4	28.2 ± 2.1	0.4
CEN4 sgR 1529	2.7 ± 0.4	10.0 ± 0.1	0.3
CEN4 sgR 1922	1.9 ± 0.1	12.4 ± 13.9	0.2
Empty vector	1.8 ± 0.5	14.6 ± 14.4	0.3

From these calculations it became apparent that the parasites' survival might be a bigger bottleneck to overcome than the propensity of surviving parasites to be transfected. This likely holds true even with the aforementioned limitation that the survival might be underestimated by my calculations.

One limitation which became apparent is the growth behavior of the parasites during these experiments. We expected to see a continuous increase in growth rate until exponential growth within one to two cycles after the point where all parasites not carrying plasmids had been cleared (cycle 3). However, exponential growth set in much later, during cycles 11-13 (Fig. 2.5 D). Fundamentally, this was not different from the cases where the GFP reporter was transfected (see data "Exponential growth detected in (cycle)" in Tab. 2.1 and violin plots in cyan in Fig. 2.3). This lag phase was characterized by parasites either not fully disappearing from thin blood smears upon addition of the selection drug (which is not unusual for *P. knowlesi*, supposedly due to the higher transfection efficiency than e.g. *P. falciparum*, where parasites disappear entirely for at least one week) or disappearing for a few cycles and then re-appearing but staying below a parasitemia of 0.5% (Suppl. Fig. 3). I hypothesized that the applied concentration of WR99210 was too high, essentially killing parasites in spite of carrying the

respective plasmid. Although I had used the drug at the recommended concentration of 2.5 nM, this was an intriguing hypothesis because it had been previously observed that when *P. knowlesi* parasites were transfected at the schizont-stage, they tended to take up only one construct during transfection, in contrast to *P. falciparum* parasites which were transfected at the ring-stage that took up several (up to five) plasmids per cell (Carrasquilla *et al.*, 2020). It did not become clear if this effect was due to the parasite strain or the applied transfection method. However, a lower plasmid copy number could likely equal lower levels of dihydrofolate reductase conferring the resistance, so that I wanted to ensure that the concentration of WR99210 was, in fact, appropriate for the dCas9 strain. I determined the drug's IC₅₀ for our dCas9 strain that was sensitive to WR99210 as well as a resistant one that had previously been transfected with the empty vector (Fig. 2.5). The resistant strain had an IC₅₀ of 20 nM, well above the concentration used for selection. In contrast, the sensitive strain had a lower IC₅₀ of 0.2 nM, meaning that these cells are efficiently killed by the 2.5 nM concentration of WR99210 we routinely used. From this, it was not possible to exclude completely that the growth lag phase was due to a selection process for parasite cells that carried more than one plasmid, but we reasoned that we could lower the WR99210 concentration from 2.5 to 2.0 nM (10x the sensitive strain's IC₅₀). Although we used this lower concentration in following transfections, the growth lag-phase persisted.

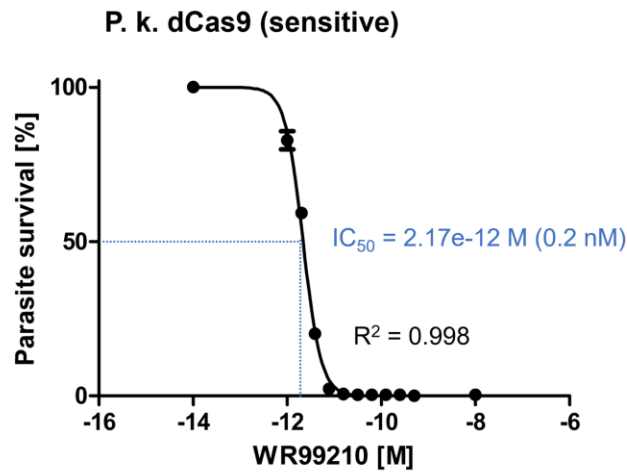
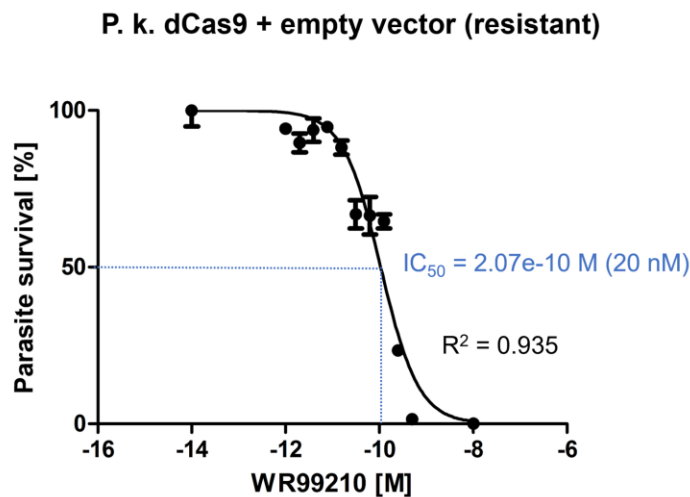
A**B**

Fig. 2.5: Determining the IC_{50} of WR99210 with dCas9-expressing parasite lines that are sensitive or resistant to the drug. The dCas9-expressing parasite strains either sensitive (**A**) or resistant (**B**) to WR99210 were grown in a 96-well plate and treated with varying concentrations of WR99210 in duplicate. After 3 cycles the parasitemia was determined via flow cytometry and IC_{50} values were calculated.

Without further inquiry into this phenomenon, it is impossible to conclude if the lack of growth was due to a form a dormant state the parasites resided in or if a steady state between growth and death of parasites existed. In any case, this limitation should be kept in mind when evaluating the transfection efficiency assessed here.

2.3 Selection of high quality sgRNAs

As described in the Introduction (see chapter 1.6), sgRNAs can mediate disruption or interference of gene expression to varying degrees, depending on several factors. One factor that positively correlates with sgRNA activity or efficiency is protospacer proximity to the TSS of the target gene. Interestingly, there seems to be some variance regarding the optimal position in relation to the TSS in different organisms (Gilbert *et al.*, 2014; Smith *et al.*, 2016). Not only are such design rules for highly efficient sgRNAs unfortunately unknown in *Plasmodium*, but also TSS annotation has only been performed for *P. falciparum* (Adjalley *et al.*, 2016) but not for *P. knowlesi*.

2.3.1 RNA-sequencing for transcription start site estimation

In preparation for this project, Ann-Kathrin Mehnert prepared *P. knowlesi* A1-H.1 WT samples for RNA-sequencing (RNA-seq) to enable TSS estimation (A.-K. Mehnert, MSc thesis, 2019). She extracted total RNA from an asynchronous parasite culture that was subsequently sent to Prof. Siegel's lab at the Ludwig Maximilian University in Munich. It was decided to perform ribosomal RNA (rRNA) depletion as described previously (Kraus, Brink and Siegel, 2019) instead of poly(A) enrichment to avoid 3' bias in the RNA-seq sample. For the rRNA depletion, Ann-Kathrin designed and subsequently sent biotinylated oligonucleotides for *P. knowlesi*-specific rRNA genes together with the samples. The Siegel lab then performed strand-specific library preparation and two technical replicates of Illumina-based RNA-seq with these.

The resulting RNA-seq reads were later analyzed by Nathan Ribot to estimate transcript levels using the RNA-seq analysis tool Cufflinks (Trapnell *et al.*, 2012). Interestingly, he found that only 11% of genes had a transcription level higher than 70 fragments per kilobase million (FPKM) and the majority (66%) of genes was expressed between 0-30 FPKM (Fig. 2.6 A). He then used the tool StringTie (Pertea *et al.*, 2015) to assemble all RNA-seq reads upstream of genes from the *P. knowlesi* H genome (at that point in time, there was no genome annotation available for the A1-H.1 strain) into full transcripts. The tool identified 5' UTR estimates as well as putative TSSs. This way, a total of 1798 putative TSSs upstream of 5483 genes annotated for the H strain genome could be estimated (Fig. 2.6 B). Since Nathan Ribot found that the 5' UTRs were more likely to be identified if they were located upstream of genes with a higher FPKM value we suspected that the number of reads mapping to the remainder of the genes was not sufficient for StringTie to identify 5' UTRs with high confidence.

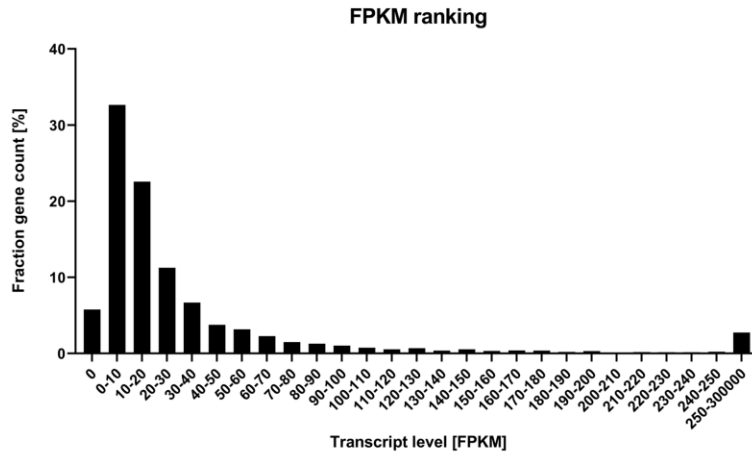
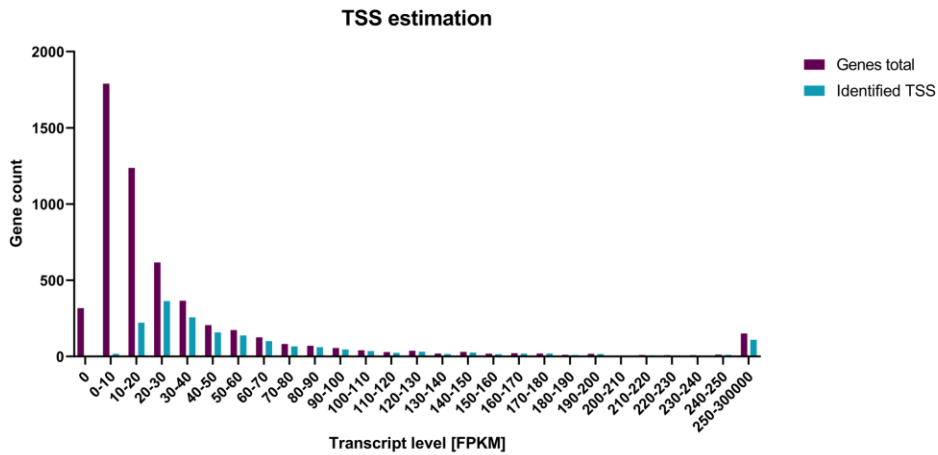
A**B**

Fig. 2.6: The majority of *P. knowlesi* genes from our RNA-seq sample are expressed at low transcript levels. (A) RNA-seq reads were analyzed with Cufflinks to estimate their transcript levels. The fraction of genes with a given fragment per kilobase million (FPKM) level are shown in steps of 10 FPKM (except the last increment). (B) StringTie was then used to assemble RNA-seq reads upstream of genes into transcripts, to estimate 5' UTRs and TSSs. The number of TSSs the program identified (turquoise) are shown per FPKM increment next to the gene count of that FPKM increment.

To identify TSSs for more genes, Nathan extracted sequences flanking the TSSs identified with StringTie (100 bp or 400 bp flanking the TSS, respectively) and then identified recurring motifs in these flanking sequences using the tools DREME and MEME (Bailey and Elkan, 1994; Bailey, 2011) (Suppl. Tab. 1). Although the identified sequences showed very good statistical significance as determined by DREME and MEME, respectively, they could not be identified in all sequences and were found to occur in CDS when mapped to the *P. knowlesi* H genome

using the tool FIMO (Grant, Bailey and Noble, 2011). Therefore, we concluded that, unfortunately, they cannot be used to identify TSSs in this sample.

2.3.2 Generation of genome-wide list of suitable sgRNAs

To facilitate the selection of high efficiency/high activity sgRNAs for CRISPRi in *P. knowlesi*, Nathan Ribot generated a list of all available sgRNA sequences targeting the 5' UTR upstream of the canonical -NGG PAM of 5358 out of the 5502 genes (including non-coding RNA genes and pseudogenes) in the *P. knowlesi* H genome (97.4% of gene coverage). For this, he used the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT), a web tool that detects all possible sgRNA sequences in a (genomic) sequence that the user provided (Peng and Tarleton, 2015). For the output, EuPaGDT uses a series of parameters to rank the sgRNAs, i.e.,

- I) a target score,
- II) the predicted sgRNA activity and
- III) microhomology-pairs flanking the sgRNA cut site.

The target score considers how well a given sgRNA can target an on-target sequence(s) while avoiding off-target sequence(s). The predicted sgRNA activity is based on empirical data from a study which investigated the effect of the nucleotide composition of the 20 nt targeting sequences as well as 4 nucleotides up- and 3 nucleotides downstream of this sequence on the sgRNA's activity (Doench *et al.*, 2014). Although the results originated from a mammalian cell system, EuPaGDT's authors reported that high activity scores from Doench *et al.* correlated with effective sgRNA function in the parasite *Trypanosoma cruzi* (Peng and Tarleton, 2015). EuPaGDT provides a multi-threaded batch mode for processing genome-scale gene lists which was very useful for my purposes. Regarding microhomology-pairs flanking the sgRNA cut site, it can be important to consider them when designing sgRNAs for CRISPR-Cas9 since microhomology-mediated end-joining of the introduced DSB has led to local sequence deletions in mammalian cells as well as eukaryotic parasites (Peng *et al.*, 2015). For my purposes, microhomology pairs were not of particular importance since I employed a dCas9 and therefore no DSBs were introduced.

As mentioned earlier, Adjalley *et al.* found that the majority of TSSs are located within 1 kb upstream of CDSs in *P. falciparum* (Adjalley *et al.*, 2016). In accordance with this, Nathan Ribot found that this was also the case for the predicted TSSs in our *P. knowlesi* sample (Fig. 2.7 A). To make the sgRNA list as comprehensive as possible while excluding sgRNAs that targeted too far away from the respective TSS, and thus likely exhibiting low activity, it was decided to

compile the list with sgRNAs targeting within 2 kb upstream of CDS of genes. To illustrate this, Figure 2.7 B shows sgRNAs targeting ca. 2 kb upstream of the CDS of an example gene coding for a Dna J protein (PKNH_1120300). Subsequently, Nathan Ribot applied filters to the list, thereby removing sgRNAs with a GC-content below 30%, a CRISPRater score below 0.59 and a total score below 0.1, the latter being an unweighted score of all available scores (Suppl. Tab. 2). Furthermore, all sgRNAs that had either a perfect or imperfect match for an off-target hit were removed which resulted in a list of nearly 223 000 sgRNAs. To give each sgRNA a unique identifier, he chose a compound ID comprising the gene's ID (e.g., PKNH_1120300) and the number of bp starting 2 kb upstream of the gene's CDS. Therefore, PKNH_1120300_1900 would be a sgRNA binding 100 bp upstream of the CDS of PKNH_1120300. To make it clearer, I changed the gene ID for the sgRNAs used in this thesis to the gene's name but kept the numerical bp value, e.g. "Dna J sgRNA 1900".

This sgRNA list represents an invaluable tool for designing or selecting sgRNA sequences and I anticipated that it would be very useful for compiling a sgRNA library, especially for high-throughput screens.

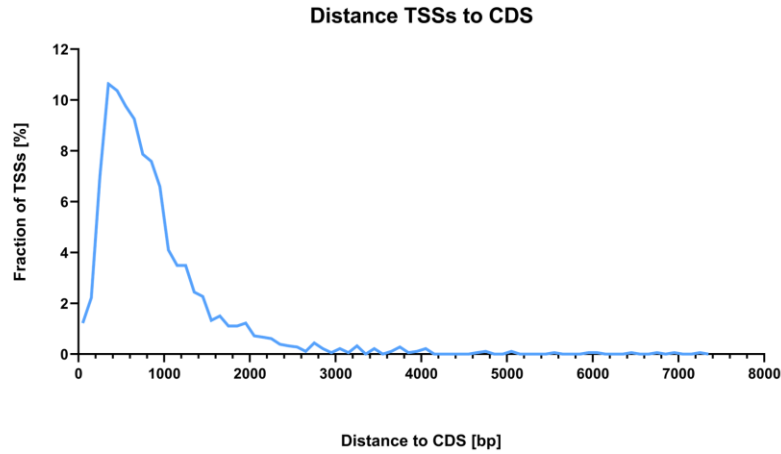
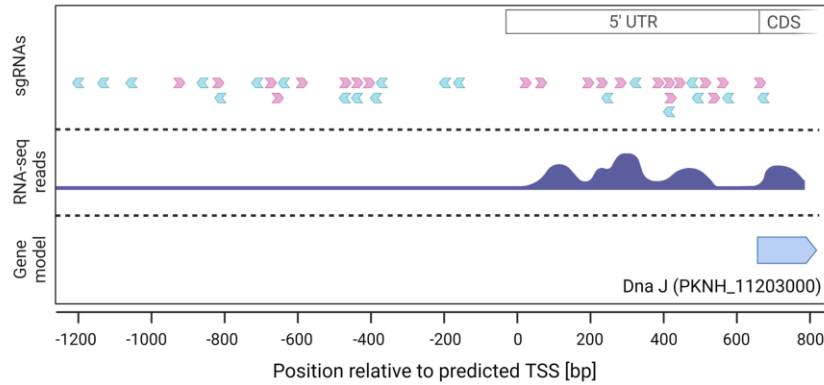
A**B**

Fig. 2.7: Proximity of sgRNA sequence to the TSS of the target gene is an important indicator for high sgRNA activity/efficiency. (A) The distance of TSSs estimated previously to the respective coding sequence (CDS) of the RNA-seq data set was determined *in silico* and the fraction of TSSs in relation to their distance to CDS in bp is shown here. (B) Schematic of an exemplary genomic locus upstream of a Dna J CDS (PKNH_11203000) (gene model in blue on the bottom). RNA-seq reads (purple) and all possible sgRNA sequences (pink and cyan, depending on orientation) were mapped to that region.

2.4 Validation of knockdown efficiency

A self-evident pre-requisite for the success of a CRISPRi screen is the ability to show specific and efficient knockdown of target genes. Being able to demonstrate knockdown of essential genes, for cell culture-maintained strains in the asexual blood-stages, would be particularly powerful since targeting these genes by knockout approaches is inherently impossible and has made their systematic functional analysis mostly elusive so far.

2.4.1 Optimization of the pL6-opti-library plasmid backbone

As mentioned previously, library generation for this CRISPRi screen approach includes cloning the 20 nt-long spacer oligomers into the pL6-opti-library backbone in a pooled fashion as described in (Joung *et al.*, 2017). The pL6-opti-library plasmid is based on the pL6-opti-tracr plasmid which was kindly provided by Prof. Artur Scherf from Institut Pasteur in Paris. Prior to this PhD project, it had been optimized for usage in *P. knowlesi* by exchanging the *P. falciparum*-based hDHFR selection cassette with the respective *P. knowlesi*-based counterpart from the pPkconGFP plasmid. For expression of the sgRNA, it contains a sgRNA expression cassette with U6 5' and 3' UTRs and an optimized tracrRNA sequence. For cloning of the sgRNA, the backbone was linearized using the *BtgZI* restriction enzyme, gel-purified and the sgRNA sequence with overhangs was cloned in via two-fragment Gibson assembly. To ensure efficient cloning with the *BtgZI* enzyme, the plasmid needed to be digested for 3 h at 65°C and the linearized backbone needs to be gel-purified twice which made the process more laborious and time-consuming than using other restriction enzymes. Therefore, I replaced the *BtgZI* restriction sites downstream of the 5' UTR and upstream of the tracrRNA sequence with *BbsI* sites. In our hands, using the *BbsI* enzyme was 2.5x faster and 20x more efficient than using *BtgZI*.

Since the regulatory regions of the pL6-opti-library sgRNA expression cassette were also based on *P. falciparum*-specific sequences, I wanted to replace the U6 5' UTR with a *P. knowlesi* codon-optimized version from the plasmid pCas9-sg_no-guide, provided by the Moon lab (Mohring *et al.*, 2019), which was unfortunately not successful. Even after several attempts to PCR-amplify the 1244 bp long region of interest from pCas9-sg_no-guide and cloning it into pL6-opti-library, I did not obtain a plasmid with the correct sequence (data not shown). It is likely that it was not possible to obtain a complete and correct sequence for the U6 5' UTR due to its highly repetitive nature since the AT-content is relatively balanced at 67% for a *plasmodial* non-coding region.

2.4.2 Validation of knockdown efficiency by transcriptional analysis and growth assays

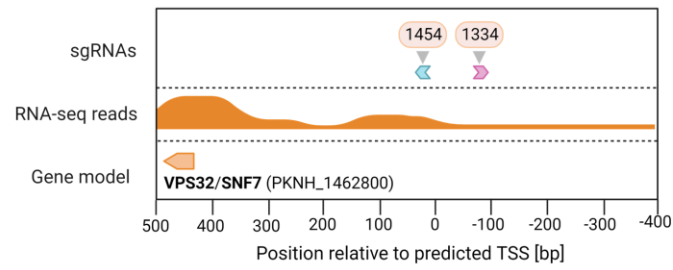
For validating knockdown efficiency, I initially chose a gene that is essential in the asexual blood-stages, which codes for the vacuolar sorting protein Vps32 (also called Snf7). For this gene, Ann-Kathrin Mehnert had already selected two sgRNA sequences (sgRNA 1454 and sgRNA 1334) based on EuPaGDT efficiency scores and cloned them into the pL6-opti-library plasmid. Figure 2.8 A schematically shows where the respective sgRNA sequences align in the 5' UTR of the VPS32 gene. The sgRNA 1334 served as a knockdown control since its sequence not only yielded low efficiency scores but a point mutation had accidentally been introduced during

cloning. Ann-Kathrin transfected these constructs and an empty vector control into the dCas9 B5 background but Western blotting analysis of the Vps32 protein level in the respective knockdown strains compared to an empty vector control remained inconclusive because no signal for the Vps32 protein could be detected.

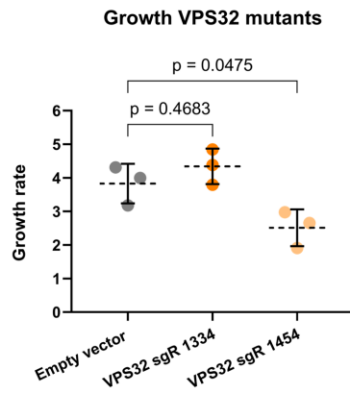
Instead of checking the protein level, I decided to perform growth assays with knockdown strains since the knockdown of an essential gene is likely to produce a growth defect. For this, I transfected the sgRNA constructs again, this time into the dCas9 F6 clone, suspecting that the low transfection efficiency could be specific to the B5 clone. The parasitemia was determined by taking images of thin blood smears with a Keyence tabletop microscope. While I counted infected RBCs by eye, the total number of RBCs was detected and counted by the software for this microscope. This is done using a mask that can be customized to fit the conditions of the smear, such as degree of staining (which influences contrast) or confluence of cells (Suppl. Fig. 4). In the growth assay, a growth defect ($p=0.05$) in the strain transfected with sgRNA 1454 compared to the empty vector control (Fig. 2.8 B) was revealed. As expected, the knockdown control parasite line (sgRNA 1334) did not show a growth phenotype. A second technique to validate a knockdown is to analyze the transcript levels of the target gene using RT-qPCR. Unfortunately, there is no comprehensive transcriptomics data available for *P. knowlesi* parasites to date but, at least in *P. falciparum* 3D7 parasites, the VPS32 gene shows a stage-specific transcript expression profile, with the highest transcript levels present in the schizont-stage (especially 40-48 hpi) (Suppl. Fig. 5). We continued under the assumption that the general expression profile in *P. knowlesi* is similar to *P. falciparum*, so I synchronized parasites with Nycodenz gradient centrifugations in two consecutive cycles before harvesting schizont-stage parasites (while parasites grow synchronously inside the human host, this synchrony is lost in cell culture). After I had extracted RNA and produced cDNA from the samples, I analyzed transcript levels in the strains and found that the reduction in transcript levels in both the knockdown control parasite line (sgRNA 1334) as well as the line that received sgRNA 1454 was not statistically significant compared to the empty vector control (Fig. 2.8 C). One explanation for this outcome is that a minor reduction in VPS32 transcript levels might have been sufficient to induce a significant growth defect in the sgRNA 1454 knockdown line. This is supported by the fact that the VPS32 gene has a very low MIS of ca. 0.12 as determined in the genome-wide *piggyBac* knockout screen performed in *P. falciparum* (Zhang *et al.*, 2018) (see chapter 1.5). To recapitulate, if the MIS of a particular gene is close to 0, parasites that had been recovered in the screen had none or almost no insertions in the CDS of this gene and therefore the gene is assumed to be essential.

Next, I chose to target the non-essential centrin-4 (CEN4) gene by transfecting three sgRNA constructs separately. As reported earlier, the selection of sgRNA sequences was mainly based on overall proximity to a putative TSS and the highest possible GC-content of the sgRNAs. Since the 5' UTR of the CEN4 gene did not show an obvious TSS I decided to select sgRNAs at three locations: sgRNA 1922 just upstream of the start of the CDS, sgRNA 1529 just upstream of what could be the (main) TSS and sgRNA 1128 just upstream of what could be a secondary TSS (fewer reads mapped here compared to where sgRNA 1529 binds) (Fig. 2.8 D). As expected from targeting a gene that is non-essential in the asexual blood-stage, there was no difference in growth detectable in the knockdown cultures compared to the empty vector control (Fig. 2.8 E). For transcript analysis by RT-qPCR, I again synchronized the parasites to harvest schizonts as before since CEN4 is also expressed mainly at this stage (Otto *et al.*, 2010) (Suppl. Fig. 5). Interestingly, I found a significant >50% reduction in CEN4 transcripts in the CEN4 sgRNA 1529 culture compared to the empty vector control while the reduction of transcripts in the other two knockdown cultures was not significant (Fig. 2.8 F). This suggested that sgRNA 1529 indeed mediated a knockdown of the CEN4 gene and that the genomic location where RNA-seq reads increased just downstream of its binding site in the 5' UTR could be the main TSS of the gene.

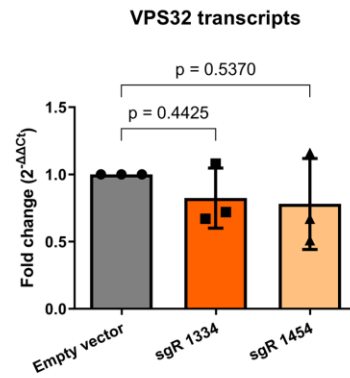
A



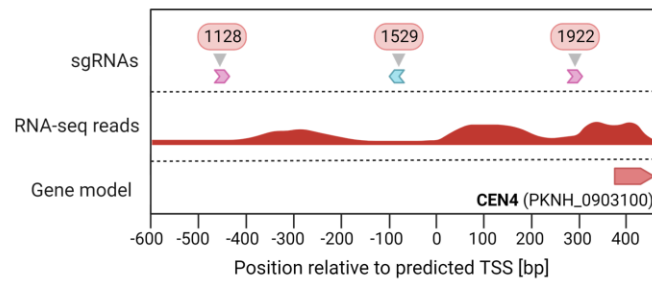
B



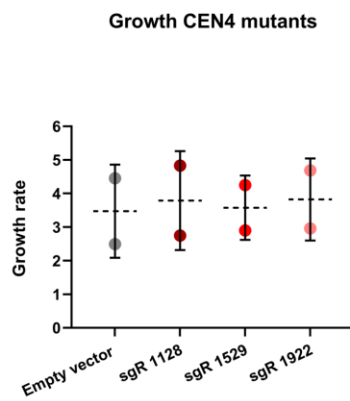
C



D



E



F

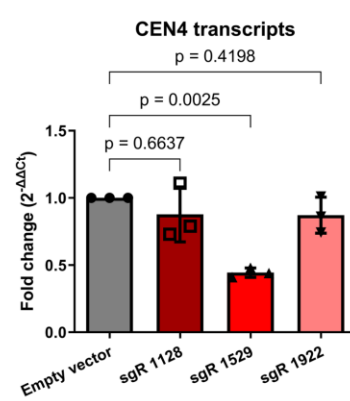


Fig. 2.8: Validation of knockdown efficiency targeting the essential VPS32 and non-essential CEN4 genes. (A) and (D) Schematic representation of the gene model, RNA-seq reads and selected sgRNAs for the 5' UTR of the VPS32 (A) and CEN4 (D) genes. These panels were created with BioRender.com. (B) and (E) Parasitemia of parasites transfected with sgRNAs targeting VPS32 (B) or CEN4 (E), as well as an empty vector control, were monitored for 2 cycles and their growth rate was calculated. In (B), three biological replicates were performed and one-way ANOVA with Dunnett's multiple comparisons test was performed to test significance of the results. (C) and (F) Using parasites synchronized to the schizont-stage, total RNA was extracted from which cDNA was produced and transcript levels of the VPS32 gene (C) and CEN4 gene (F) were measured by RT-qPCR. Each symbol is a biological replicate, with the bar representing the mean value and the error bars the standard deviation. The significance was tested by one-way ANOVA with Dunnett's multiple comparisons test.

Based on these results, I wanted to further validate the capacity of the system to mediate knockdown and chose to target another non-essential gene, coding for spindle assembly abnormal protein 6 (SAS6). The RNA-seq reads for its 5' UTR suggested the location of the TSS to be ca. 600 bp upstream of the CDS although the number of reads was also moderate here (Fig. 2.9 A). A steeper increase in reads could be identified <200bp upstream of the CDS which could also be a secondary TSS. I selected five sgRNA sequences both up- and downstream of this putative TSS located ca. 600 bp upstream of the CDS, one of them located just upstream of this putative secondary TSS (sgRNA 1672). By targeting the SAS6 gene with five instead of three sgRNA constructs, I hoped to start deducing some rules as to what constitutes a high-quality sgRNA in *P. knowlesi*.

Unfortunately, there was no knockdown observable on the transcript level in any of the knockdown cultures (Fig. 2.9 B). A possible explanation for why no knockdown could be detected by targeting the SAS6 gene is that its expression level is relatively low to begin with (ca. 50 transcripts per million (TPM) for SAS6 compared to ca. 100 TPM for CEN4 and ca. 170 TPM for VPS32; all at schizont stage) (Suppl. Fig. 5).

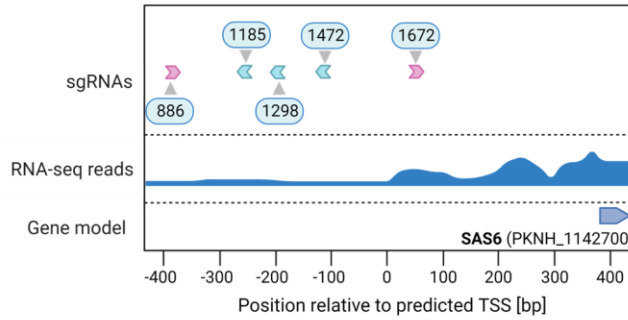
A**B**

Fig. 2.9: Validation of knockdown efficiency targeting the non-essential *SAS6* gene. (A) Schematic representation of the gene model, RNA-seq reads and selected sgRNAs for the 5' UTR of the *SAS6* gene. This panel was created with BioRender.com. (B) Using parasites synchronized to the schizont-stage, total RNA was extracted from which cDNA was produced and transcript levels of the *SAS6* gene were measured by RT-qPCR. Each symbol is a biological replicate, with the bars representing the mean and the error bar the standard deviation.

Since the generation of knockdown cultures and performing experiments for both knockdown validation and assessment of transfection efficiency were laborious and were impeded by some technical difficulties, it was decided at this point to shift the focus to performing a small-scale screen.

2.5 Proof-of-principle small-scale knockdown screen

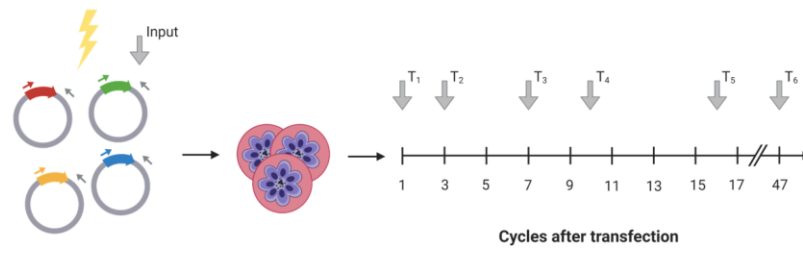
The rationale for performing this small-scale screen was severalfold:

- (I) It was intended to serve as a proof-of-principle experiment to demonstrate that, using this setup, it is possible to
 - a. transfect several **sgRNA constructs at uniform abundance** as a pool into parasites and
 - b. follow the **dynamic changes in their abundance** over time.
- (II) The screen was designed as a so-called drop-out screen, where sgRNAs that confer an efficient knockdown of an essential target would disappear over time from the parasite population which would strongly indicate the **functionality of knockdown by CRISPRi in *P. knowlesi*** and its application in a screening setting.
- (III) Further validate knockdown efficiency since the screen would indicate which sgRNA(s) mediated an efficient knockdown. Thus, **more targeted validation experiments** with these sgRNAs and selected control sgRNAs would be possible.

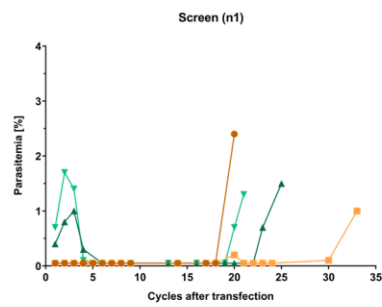
2.5.1 The small-scale proof-of-principle screen revealed that no efficient and specific knockdown conferred by CRISPRi could be detected

Since the parasites carrying different sgRNA constructs would be in direct growth competition with each other after transfection, I hypothesized that the detection of differences in growth originating from knockdown would be detectable more sensitively than in the previously performed growth assays where those parasites existed as distinct parasite lines and therefore also grew as separate cultures. I decided to use RT-qPCR to monitor the relative abundance of each sgRNA construct at different timepoints after transfection. The library size of 20 sgRNA constructs was sufficiently small so that I was able to design specific RT-qPCR primers for each construct. The constructs were identical except for the unique 20 nt spacer sequence, meaning that the primers had to be designed to bind these sequences so that the constructs became unequivocally identifiable. Since it was crucial to ensure the best possible binding behavior and overall performance of the primers for this experiment, I carefully analyzed all sequences for possible hairpin structures as well as homo- or hetero-dimers which led to minor nucleotide changes in some of the primers (see Tab. 4.13 in chapter 4.1.8). With the help of these primers, I was able to use RT-qPCR as a readout to quantitatively assess the changes in sgRNA construct abundance at specific, premeditated timepoints (Fig. 2.10 A).

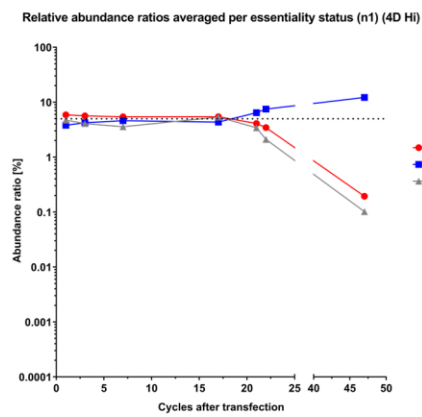
A



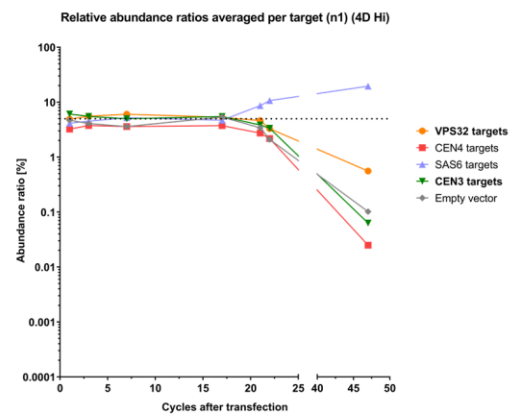
B



C



D



E

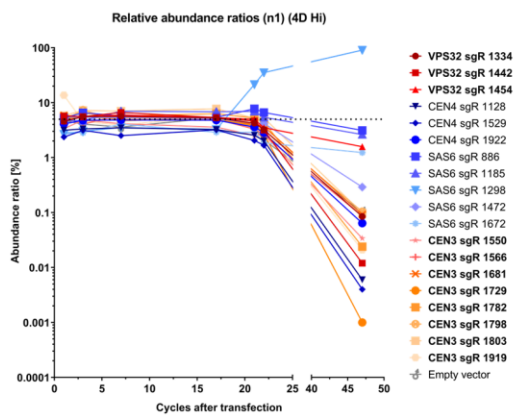


Fig. 2.10: Proof-of-principle screen comprising 20 sgRNAs and targeting essential (VPS32, CEN3) and non-essential (CEN4, SAS6) genes. (A) Schematic of the experimental strategy for the screen. Plasmids were transfected into segmented schizont-stage parasites (each plasmid schematic represents one of the four targets). Samples of the input and of several timepoints after transfection were taken (grey arrows). This panel was created with BioRender.com. (B) Growth of the 4 transfected cultures (i.e., 4 conditions). Parasitemia was counted on thin blood smears. (C)-(E) From the parasite samples, DNA was extracted and used for RT-qPCR to determine the constructs' abundance. The Ct values were used to calculate relative abundance ratios for each construct which represents its relative abundance in the plasmid pool as a percentage. Since there were 20 constructs in the pool, if all the constructs were present at uniform abundance, the relative abundance ratios approximated 5% (dotted line). sgRNA IDs for sgRNAs targeting essential genes are shown in bold in the legends. The abundance ratios were averaged per essentiality status of the target (C) or per target gene (D) for a better overview. (E) The abundance ratios of all 20 constructs are shown and color-coded for essential targets (red/orange) and non-essential targets (blue).

The constructs in the library targeted four genes in total: the essential VPS32 and CEN3 genes as well as the non-essential CEN4 and SAS6 genes (Tab. 2.3). In the first replicate of the screen the library was transfected using four different conditions: with the 4D and the 2b nucleofector and using a high ("Hi") or low ("Lo") plasmid amount, respectively (Fig. 2.10 B). This was done to increase the probability that at least one condition resulted in an efficient transfection outcome.

Tab. 2.3: Protospacer sequences that were cloned into library plasmids to target VPS32, CEN4, SAS6 and CEN3. Gene IDs are given for the *P. knowlesi* H strain (PKNH) and the A1-H.1 strain (PKA1H). sgR: short for sgRNA. NA: Not applicable.

Target	Gene ID	sgRNA ID	Protospacer sequence
VPS32/SNF7	PKNH_1462800, PKA1H_140068900	VPS32 sgR 1334	TTTGTGTTTATACCTGGATTTA
		VPS32 sgR 1442	TTGTGACAATTTGCGCAACT
		VPS32 sgR 1454	GCGCAACTTGGTTCCCCCTC
CEN4	PKNH_0903100, PKA1H_090008600	CEN4 sgR 1128	TGGGGGCACGTCATCACCTC
		CEN4 sgR 1529	GCACAAATGTGTCACAAAGG
		CEN4 sgR 1922	TGCTGAATGCTTAGCAAACG
SAS6	PKNH_1142700, PKA1H_110048900	SAS6 sgR 886	AAGGAGGAGCCCCGCGGAAA
		SAS6 sgR 1185	AGCGCGCGAAACTGCCGCAC
		SAS6 sgR 1298	GGTGAGATTCTCCTTCAAGC
		SAS6 sgR 1472	TTGCACGAAAACCTCCACAGG
		SAS6 sgR 1672	ACGCGGTAGACGGAGCACTG
CEN3	PKNH_0612100, PKA1H_060016900	CEN3 sgR 1550	CCGAGTGGGAAGCCTTACCG
		CEN3 sgR 1566	ACCGTGGTGCGATCAGCCCA
		CEN3 sgR 1681	GATATACTGCTCGTGCGTAA

		CEN3 sgR 1729	CAAGAAGTCACTAATTCGAG
		CEN3 sgR 1782	GGACTGGGGAGATAAAGAAG
		CEN3 sgR 1798	AAAGAGCTCGTGATGCGGAC
		CEN3 sgR 1803	AAAGTAAAGAGCTCGTGATG
		CEN3 sgR 1919	GCTCGAAAGCGTGCACACAC
NA	NA	Empty vector	NA

In all four conditions the plasmid mixture derived from the same stock which was in turn also used to measure the threshold cycle (Ct) values of each plasmid and subsequently calculate the relative plasmid abundance in the “Input” sample by RT-qPCR. For this calculation, the abundance of each specific plasmid was first normalized to the abundance of all plasmids using a primer pair that bound in the hDHFR resistance cassette (primer pair 21 and 22, Tab. 4.13 in chapter 4.1.8) present in all sgRNA constructs. From this, the relative abundance ratio of each sgRNA construct was calculated as follows:

$$\Delta Ct = Ct(specific) - Ct(hDHFR)$$

$$Relative\ abundance\ ratio = 2^{-\Delta Ct}$$

Since there were 20 different sgRNA constructs in the library, if they were all present at equal abundance, their relative abundance ratio would be 5%. The measured relative abundance ratio of each plasmid in the input was very uniform (mean: 4.91% $\pm$ 0.97% SD) (Suppl. Fig. 6) and therefore, one of the prerequisites for a meaningful readout of the screen was met. The ratios for the timepoints after transfection were obtained in a similar way after DNA was extracted from the respective parasite culture sample.

As was previously observed, the nucleofection survival was much higher using the 2b nucleofector compared to the 4D nucleofector, demonstrated by higher parasitemia in the first cycles following transfection (Fig. 2.10 B). However, there was a relatively long growth lag phase following selection with WR99210 until at least 17 cycles after transfection, visible for all conditions. Since the “4D Hi” and the “2b Lo” condition reappeared in culture only one cycle apart it was reasoned that the plasmid amount and the nucleofector machine did not have a marked influence on the overall transfection efficiency.

The relative abundance of plasmids in the “4D Hi” condition was followed for 47 cycles and there were no changes in relative abundance detectable for the first 17 cycles (Fig. 2.10 C, D, E). After analyzing the other conditions, we found that similar observations could be made

(Suppl. Fig. 7 and 8). I will describe how we approached this phenomenon in the next subchapter (see chapter 2.5.2).

To get an overview of the screen's results I plotted the relative abundance ratios of the constructs averaged per essentiality status of their target, meaning averaging the ratios of all constructs targeting the essential genes and the non-essential genes, respectively (Fig. 2.10 (C), (D) and (E); constructs targeting essential genes in bold). While it was expected that, on average, the relative abundance ratios of the constructs targeting the essential genes decreased and those targeting non-essential genes increased over time, it was unexpected that the empty vector control also disappeared quickly since there should not be any selective pressure on this construct (Fig. 2.10 C).

Next, I analyzed the relative abundance ratios of the constructs averaged per target gene and found that the constructs targeting the essential genes CEN3 and VPS32 disappeared from the population, as expected (Fig. 2.10 D). Interestingly, the constructs targeting CEN4 disappeared as well, which could still be conceivable, in general, although they are targeting a non-essential gene since the constructs' abundance is quantified relatively to each other and one single sgRNA construct (SAS6 sgRNA 1298) took over the population relatively quickly after cycle 17 (Fig. 2.10 E). However, it was unexpected that the constructs targeting CEN4, on average, disappeared from the population even faster than the constructs targeting CEN3 and VPS32 which further increased my suspicions that the results of the screen might not reflect my expectations.

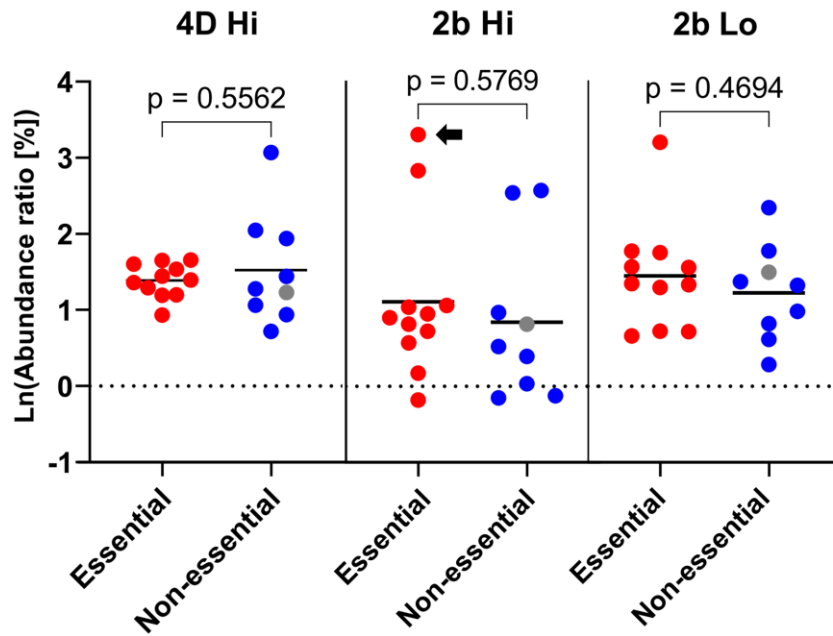
To monitor individual constructs, I plotted the relative abundance ratios for all constructs color-coded according to the essentiality status of their target: non-essential targets in blue and essential targets in red/orange (Fig. 2.10 E). I expected to see a pattern where most of the blue lines would cluster to the top and the red lines to the bottom with a mixed transition around the dotted line at 5% which represents the state in which all constructs are equally abundant in relation to each other. However, there was no clear pattern to the distribution of lines in the graph. The result that was the most unexpected was that, among all constructs targeting an essential gene, VPS32 sgRNA 1454 was the construct that disappeared the slowest. In fact, its relative abundance ratio decreased slower than most constructs targeting non-essential genes which is the opposite of what I expected since this particular sgRNA elicited a slight but significant growth defect in the knockdown validation (see chapter 2.4.2).

At this point, I deemed it possible that the dominance of SAS6 sgRNA 1298 on one hand and the disappearance of the remaining constructs on the other hand were random events and not

underlying selection by knockdown of the respective genes in the parasite. To test if the changes in relative abundance of the different constructs were indeed random, I wanted to find out if there was a significant difference between the ratio values of constructs targeting essential or non-essential genes, respectively. For this, I plotted the natural logarithmic of the abundance ratio values of each construct calculated for the latest timepoint that all conditions had in common to have the maximum difference between them (Fig. 2.11). It turned out that the difference between those values of constructs targeting essential or non-essential genes, respectively, was indeed not significant in neither all three conditions of the first replicate, nor in the two conditions of the second replicate of the screen (Fig. 2.11 A and B). In the “2b Hi” condition of the first replicate, VPS32 sgRNA 1454 was even the construct that dominated in the population at the last timepoint (parasite cycle 22) (Fig. 2.11 A; black arrow) which underlined that, unfortunately, the growth defect phenotype from the knockdown validation experiments cannot be only explained by the target being transcriptionally repressed by CRISPRi (see chapter 2.4.2).

A

First replicate



B

Second replicate

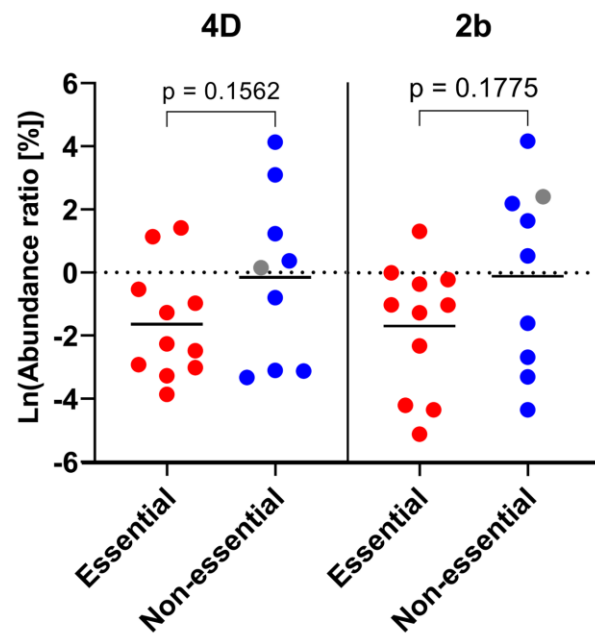


Fig. 2.11: The difference of relative abundance ratio values between sgRNA constructs targeting essential and non-essential genes was not significant in all conditions of the two replicates of the screen. (A) and (B) Relative abundance ratios of sgRNA constructs targeting essential (red) or non-essential (blue) genes from the last timepoints common to all conditions in the respective replicate of the screen (21 cycles for the first replicate (A), 27 cycles for the second replicate (B)) were transformed with natural logarithm to obtain fold-change values. These values were plotted as essential vs. non-essential target per condition (4D Hi, 2b Hi, 2b Lo for first replicate in (A) and 4D, 2b for second replicate of the screen in (B)) as a dot plot. It was tested if the difference between the two groups were significant by unpaired, two-tailed t-test. The grey dot represents the empty vector control. The dotted line represents a state where the construct neither disappeared, nor enriched in the pool. The black arrow points to construct VPS32 sgR 1454 that seemed to have elicited a growth defect in the knockdown validation (see chapter 2.4.2) but was highly enriched in the 2b Hi condition of the screen.

Taken together, there are three lines of arguments that suggest that, in the form the CRISPRi system was used here, there is, in fact, no efficient and specific knockdown detectable.

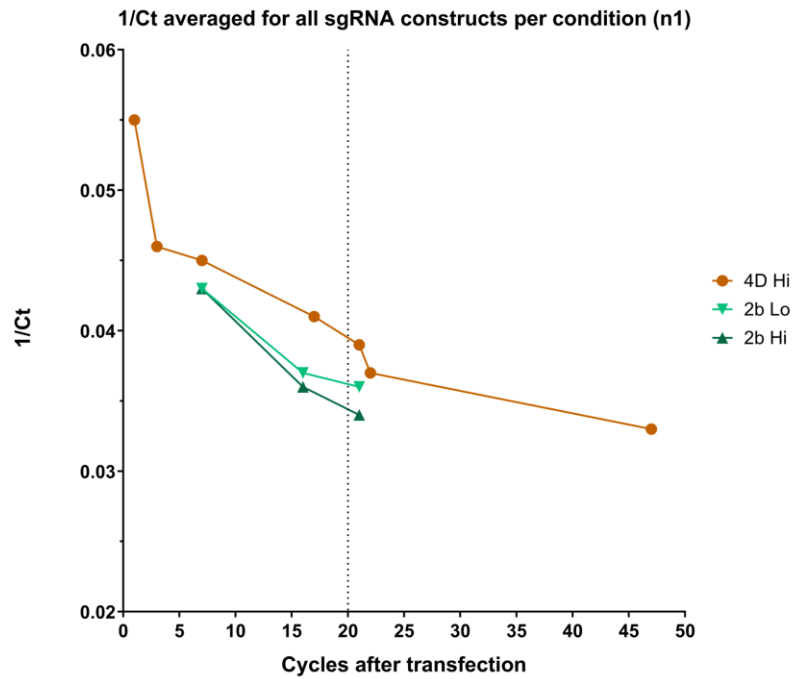
- (I) Firstly, the data obtained for VPS32 sgRNA 1454 in this screen does not support the data obtained from the growth assay during knockdown validation performed previously.
- (II) Secondly, there is no significant difference between the relative abundance ratio values of constructs targeting essential or non-essential genes in either condition of two biological replicates of the screen. Furthermore, there was no clear pattern of how these ratio values increased or decreased regarding the essentiality of the targets.
- (III) Thirdly, the empty vector control was selected against in two conditions which supports the explanation that certain sgRNA constructs were enriched (and therefore, others depleted in relative terms) by chance.

2.5.2 Examining 1/Ct values of the RT-qPCR reactions revealed excess abundance of background plasmid

Regarding the functionality of the assay itself, it seemed to serve its purpose in following the changes in relative abundance ratios of the constructs once the parasite cultures started to grow at an exponential rate (defined as before: when the growth rate was ≥ 2 -fold for at least two consecutive cycles). In fact, the time point the parasite growth curve starts to rise rapidly correlates very well with the timepoint where changes in the plasmid pool composition become apparent (Fig. 2.10 and Suppl. Fig. 9 and 10). However, it was not entirely clear why there were no changes in relative plasmid abundance visible before the exponential growth phase started. In principle, selection for parasites carrying a plasmid after transfection should have been completed during the second or third cycle (see also chapter 2.2.2), meaning I should be able to assume that from that point on all living parasites carried a sgRNA plasmid with a resistance cassette. Even if it is assumed that there is no specific and efficient knockdown mediated by the

sgRNAs expressed from the plasmids, meaning that all plasmids have the same probability of being propagated, we would have expected to see even subtle effects compound earlier. Therefore, I suspected that there was background signal from excess plasmid at the timepoints before changes in plasmid abundance became apparent. This was confirmed when I analyzed how the raw Ct values from the RT-qPCR measurements of the “4D Hi” condition of the first screen replicate developed over time (Fig. 2.12) (to make the graph more intuitive I plotted $1/Ct$ values so that a decrease in the values corresponds to a decrease in plasmid abundance). The raw Ct values decreased by 6.4 from cycle 1 to cycle 17, corresponding to a ca. 80-fold decrease in plasmid abundance while the parasitemia stayed constant during this time. From this, I speculated that washing the samples with incomplete RPMI medium before harvesting (see chapter 4.2.8) was not sufficient to eliminate excess plasmids. Furthermore, this implied that the excess plasmid was not dissolved in the medium but could be bound to surfaces, such as of RBCs that were present during the transfection. These are typically not exchanged until cycle 7 if the cultures do not need splitting beforehand. Therefore, in the second screen replicate, I treated the samples with saponin which acts as a surfactant that lyses RBCs, as well as DNase I to degrade all DNA that is not contained within the parasites before measuring construct abundance with RT-qPCR. Although I found that samples that were treated with both saponin and DNase I, respectively, showed a slightly lower plasmid abundance (inferred from a slightly higher Ct value) than untreated controls, overall, this did not mitigate the plasmid background (Fig. 2.12 B).

A



B

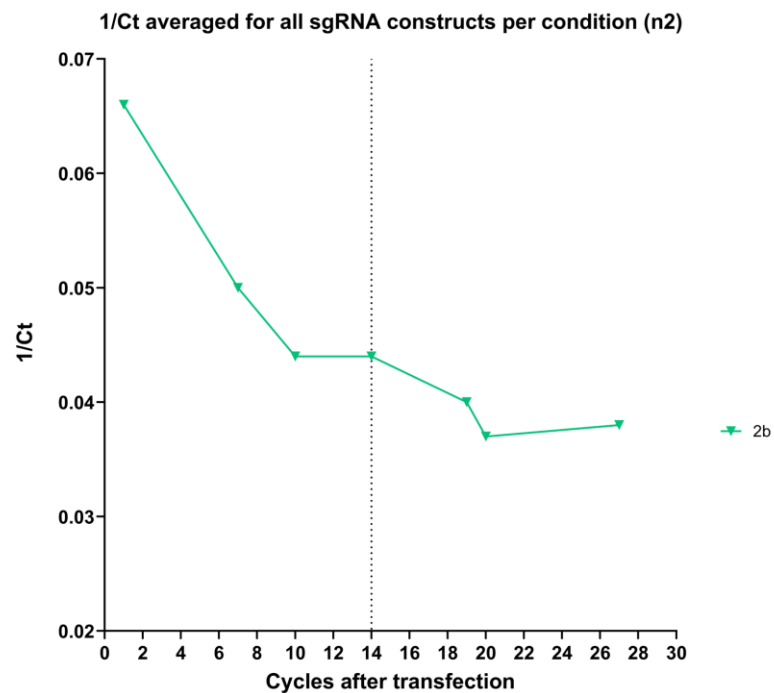


Fig. 2.12: Examining the raw Ct values from measuring the abundance ratios revealed excessive abundance of plasmid before the transfected cultures started exponential growth. (A) and (B) The raw Ct values were averaged per condition (4D Hi, 2b Hi, 2b Lo for first replicate in (A) and 4D for second replicate of the screen in (B)) and plotted as 1/Ct over the course of parasite cycles (1/Ct was chosen to make the figure more intuitive: accordingly, a decrease in 1/Ct value represents a decrease in plasmid abundance). The dotted lines indicate the timepoint exponential growth of the parasites was detected.

This outcome suggested that there is a different source for the background signal and since saponin and DNase I were not able to eliminate this excess plasmid I speculated that the source is most likely parasites that did not survive the nucleofection treatment. It is conceivable that plasmids entered these parasites that made up the vast majority of parasites present during and after transfection (97.4-99.9%) (Tab. 2.4) and that the plasmids might have been retained although the parasites did not survive the procedure. Supporting this hypothesis further is the observation that regular medium changes were not sufficient to remove these dead segmented parasites since they were still visible in thin blood smears for ca. two weeks.

Tab. 2.4: The vast majority of parasites did not survive the nucleofection reactions during the screen. The parasite death rate was inferred from the parasite survival rate that was in turn calculated as in (chapter 2.2.2). The corresponding number of dead segmented schizont-stage parasites was calculated using the number of parasites present during nucleofection (Input segmenters) that was counted as described in chapter 4.2.1.4.

Transfection reaction	Survival (%)	Death (%)	Input segmenters	Dead segmenters
Screen (n1) (4D Hi)	0,04	99,96	1,65E+08	1,65E+08
Screen (n1) (4D Lo)	0,04	99,96	1,65E+08	1,65E+08
Screen (n1) (2b Hi)	1,5	98,5	1,65E+08	1,63E+08
Screen (n1) (2b Lo)	2,6	97,4	1,65E+08	1,61E+08
Screen (n2) (4D)	0,2	99,8	8,40E+08	8,38E+08
Screen (n2) (2b)	0,9	99,1	8,40E+08	8,32E+08

Unfortunately, there was not enough time to perform further experiments to confirm that the dead parasites are indeed the source of the excess plasmid. However, if this was the case, one strategy to counteract the background signal could be to perform magnetic activated cell separation (MACS) purification of the culture after recovery from transfection. During the recovery period, healthy parasites undergo egress and invade RBCs while those that died are still present as late-stage segmented parasites. Since the latter contain the paramagnetic hemozoin crystal, they can be easily separated from newly re-invaded ring-form parasites and uninfected RBCs with magnetic columns.

3. Discussion and outlook

In this project, I aimed to establish and validate CRISPRi in the zoonotic *Plasmodium* parasite *P. knowlesi* so that it could eventually be used in a high-throughput screening approach. Part of this was to assess and optimize the transfection efficiency of the respective dCas9 strain so that complete representation of a screening library can be ensured. While I was hoping to reach previously reported transfection efficiency values of ca. 30%, an overall transfection efficiency of ca. 0.3% could be attained here. During optimization of this process, I found that using a different nucleofector than previously reported (Moon *et al.*, 2013) and using the parasite egress inhibitor ML10 seemed to help transfection outcome/success in my hands. Interestingly, I found that using the *P. knowlesi* WT strain also led to a much better transfection outcome. By examining parasite survival and the efficiency of the surviving parasites to be transfected, separately, I found that poor parasite survival seemed to be the main impediment to good overall transfection efficiency. Since the results of my knockdown validation experiments were ambiguous at first, I decided to perform a proof-of-principle knockdown screen with a library of 20 constructs targeting 4 different genes. The outcome of this screen led me to conclude that, unfortunately, I was unable to show efficient and specific knockdown with our system.

3.1 *P. knowlesi* dCas9 strain's modest transfection efficiency was mainly impeded by poor parasites' survival of nucleofection

One main reason why I decided to use *P. knowlesi* as the strain of choice for my experiments was because of its reported high transfection efficiency of up to 30-40% (Moon *et al.*, 2013; Mohring *et al.*, 2020), besides its more balanced genomic AT-content. Unfortunately, the transfection efficiencies that I was able to determine in the course of this work stayed ca. 100-fold below that with an overall efficiency of 0.3% for the dCas9-expressing strain (see chapter 2.2.2). However, I did not use the same methodology as Moon *et al.* to assess transfection efficiency, as they examined parasites expressing the reporter construct under a fluorescence microscope and counted fluorescent parasites by eye whereas I either measured fluorescent parasites with flow cytometry (see chapter 2.2.1) or used the parasites' resistance against the selection drug to calculate the parasites' survival and transfection efficiency (see chapter 2.2.2). Admittedly, reproducing the experiments the same way would have been a good opportunity to better compare the results and perhaps understand the putative discrepancy in transfection efficiency better. However, as I was able to show in two biological replicates of a transfection comprising a total of eight reactions, the parasite survival was only between 1.4-2.7% (see Tab. 2.2 in chapter 2.2.2.) and therefore constituted an important bottleneck for successful

transfection. In fact, during the 14 transfections that I performed during the work for this thesis, which comprised 39 reactions using the WT strain, as well as two clones of the dCas9 strain, I was never able to reproduce almost 2% parasitemia right after or within the first cycle after transfection (data not shown) that is presented in (Moon *et al.*, 2013). It is not unusual that the majority of cells die during electroporation, as it has been reported that typically 50-70% of cells do not survive electroporation, depending on the cell type (Kumar, Nagarajan and Uchil, 2019). Due to the previously mentioned difficulties of transfecting an intracellular parasite, this rate is likely conceivably higher using *Plasmodium* parasites. However, up to 99.9% of parasites not surviving the nucleofection reaction seems excessive. Although it has been reported that the efficiency of transfection using *P. knowlesi* decreases nonlinearly when less than 10^8 segmented schizonts are used (Mohring *et al.*, 2020), Janse *et al.* suggested the success of transfections using *P. berghei* schizont-stage parasites was less dependent on the number of parasites used but more so on their viability (Janse *et al.*, 2006). Indeed, I was not able to see a significant difference in transfection outcome/success if I used more or less than 10^8 parasites, although this investigation was not entirely systematic because for some transfections the number of parasites during the nucleofection were calculated from the recorded parasitemia and in most other cases the number of parasites were counted with a counting chamber.

Furthermore, the type of transfection, using segmented schizont-stage parasites, was chosen because it has been reported to be the superior type of transfection for *P. berghei* and the method of choice for *P. knowlesi* (Janse *et al.*, 2006; Moon *et al.*, 2013; Mohring *et al.*, 2020). However, after having considered the results presented here, it might be worth trying other transfection methods as well. These include transfection of ring-stage parasites, which might not bring about better or even worse transfection efficiencies but are less laborious regarding preparation (the parasites do not have to be synchronized as tightly) and, therefore, could be performed more often or with a higher number of parasites. Another method is often referred to as “pre-loading”, in which uninfected RBCs are transfected first and then synchronized late-stage parasites are allowed to re-invade these “pre-loaded” RBCs. This method is certainly gentler for the parasite cells and should, thus, improve their survival. In addition, it has recently been reported that pre-loading showed a better transfection efficiency in *P. falciparum* than other methods, including ring-stage and schizont-stage nucleofection (Wang *et al.*, 2022). Parameters that are often used to assess the electroporation reaction are the voltage and the time constant the electroporation machine used in a particular electroporation reaction. Unfortunately, when using the nucleofactors from Lonza Bioscience, this is not possible as these parameters are proprietary information, and the machine only gives non-numerical feedback if the transfection

reaction was adequate. This makes it also harder to compare the conditions used during transfection with other studies that did not utilize this particular machine. The same applies to the exact composition of their nucleofection solution.

While the previously mentioned overall transfection efficiency of 0.3% that I estimated using the parasites' resistance to WR99210 (see chapter 2.2.2) is my best estimation/approximation based on the experiments performed, it carries an important limitation. This limitation is a phenomenon I have observed with essentially all transfections of sgRNA constructs, namely that parasites seemed to reside in a growth lag-phase for 7-17 cycles. To find out if the concentration of the selection drug WR99210 was too high I determined the drug's IC₅₀ with a sensitive and a resistant strain. This revealed that we could, in fact, lower the applied final concentration from 2.5 nM to 2.0 nM. However, since I used a strain that had been under WR99210 selection pressure for several weeks, I could not exclude that during this time selection for parasites occurred that carried more than one copy of the plasmid with resistance cassette. To understand this process better, one could repeat the growth assay for IC₅₀ determination with a freshly transfected culture.

As mentioned in the introduction, there have unfortunately been hardly any studies that examined the process of transfection itself in *P. knowlesi*. However, recently a study was published that used both *P. falciparum* and *P. knowlesi* parasites for a screen of ca. 90 barcoded constructs (Carrasquilla *et al.*, 2020). While the authors did not specifically show or mention the transfection efficiency of their reactions, I used the parameters they defined in the paper, such as volume of schizont-stage parasites used during transfection and the number of constructs the parasites had taken up (was estimated in their paper) to estimate the transfection efficiency. This amounted to an estimated transfection efficiency for their screen between 0.00002-0.00006% for *P. knowlesi*, meaning ca. 500-15,000x lower than in the experiments shown in chapter 2.2.2 of this thesis. Intriguingly, the researchers in this study were able to show that, using 1.5×10^8 parasites, the whole library of 94 barcoded plasmids was represented in the plasmid pool despite the quite low transfection efficiency. Therefore, I concluded that, although I was not able to reach the transfection efficiency I initially aimed for, the achieved efficiency of 0.3% was adequate for screens with medium-sized libraries.

3.2 Approaches to explain contradictory knockdown results and to improve knockdown efficiency

To test knockdown efficiency, I initially performed knockdown validation experiments in which I performed growth assays monitoring the growth rate of VPS32 and CEN4 knockdown lines

compared to the line transfected with the empty vector and measured transcript levels in these cell lines. While I identified a significant growth defect in the line transfected with the VPS32 sgR 1454 construct, there was no significant reduction in VPS32 transcript levels in this knockdown line detectable. At first, we hypothesized that a modest, non-significant reduction of VPS32 transcripts might have been sufficient to cause a significant growth defect, given the gene's high essentiality status (MIS close to 0). However, at the end of the screen this construct was found among the most abundant ones in the plasmid pool in some conditions, clarifying that the observed growth defect was not trustworthy. I therefore concluded that there was an apparent discrepancy between our knockdown validation experiments and the results of the screen, meaning that I was unable to detect a knockdown that is both specific and efficient via our CRISPRi system. While it is conceivable that, in the knockdown validation, the VPS32 sgRNA 1454 line simply acquired an unrelated mutation which caused it to grow slower compared to the control, it is unclear at this point why such a prominent reduction in CEN4 transcripts was detected in the CEN4 sgRNA 1529 line.

At this point, it is difficult to assess why there was no specific and efficient knockdown detectable in the screen. However, one could speculate on scenarios that could have led to this outcome. One possible explanation is that the sgRNA constructs reached the parasite (in fact, the constructs could be detected by PCR from parasites' DNA extracts; data not shown) but only the hDHFR selection cassette was expressed and the transcription of the sgRNA cassette was selectively repressed by an unknown mechanism. This would also explain why I observed a large proportion (ca. 50%) of the parasite population that was resistant to the selection drug after transfection but did not express the reporter construct (see chapter 2.2.1). While this phenomenon of apparent selective expression cassette silencing has been observed in our lab (Voß, 2023), to my knowledge no mechanism has been described in the literature. Considering the scenario with my reporter construct, one could check if the GFP expression cassette is transcribed by using fluorescence activated cell sorting (FACS) to select the non-fluorescent but WR99210-resistant cells, extract RNA from these and perform RT-qPCR to check if GFP transcripts can be detected. This could be complemented with IFAs to check for the presence of hDHFR on these cells. In the case of the sgRNA-encoding constructs, one could perform chromatin immunoprecipitation with RT-qPCR on dCas9 and check if it can be detected occupying the genomic locus of the sgRNA's target. Unfortunately, it is not possible to couple the expression of the hDHFR cassette to the expression of the sgRNA cassette in this case because the former is transcribed via RNA polymerase II whereas the latter is transcribed via RNA polymerase III. However, implementing the RGR system developed by Walker and

Lindner to express the sgRNA, which allows sgRNA transcription by RNA polymerase II, might enable the fusion of the sgRNA expression cassette with the hDHFR selection cassette.

For the selection of sgRNAs, I adhered to what other studies using CRISPRi in yeast and human cells have reported, i.e. that the closer the sgRNAs bound to the target gene's TSS, the more efficient knockdown via dCas9 was (Gilbert *et al.*, 2014; Smith *et al.*, 2016). Another general indicator of high sgRNA activity is a balanced GC-content (Doench *et al.*, 2014) which also influenced my sgRNA selection. To learn more about what makes a sgRNA highly active/efficient for CRISPRi in *P. knowlesi*, a systematic study where every possible sgRNA tiling the 5' UTR of one or several target gene(s) could be performed as Gilbert *et al.* did with human cells (Gilbert *et al.*, 2014). For an easier readout, one could tag an endogenous locus with a reporter as was done in (Walker and Lindner, 2019) and target this locus so that a fluorescence-based readout of knockdown efficiency would be possible. Complementary to this, systematically annotating TSSs in *P. knowlesi* as it was done in (Adjalley *et al.*, 2016) for *P. falciparum* would be very beneficial for properly defining sgRNA rules in this parasite because it is very likely that also in this organism proximity to TSS of the target gene plays a role. Likewise, the systematic mapping of transcription factor binding sites might also be helpful in deciphering their role or contribution to successful targeting of dCas9.

Lastly, the absence of an efficient knockdown could also be because, under the circumstances that we used, the dCas9 was not sufficiently potent to mediate an efficient knockdown. Although a knockdown mediated by only dCas9 has been reported for *P. falciparum* (Baumgarten *et al.*, 2019; Barcons-Simon *et al.*, 2020), it has not yet been reported for *P. knowlesi*. To likely enhance the transcription downregulation effect, one could use dCas9 coupled to the histone deacetylase domain Sir2a, as was done in *P. falciparum* (Xiao *et al.*, 2019; Liang *et al.*, 2022). While a dCas9-Sir2a fusion protein likely amplifies the downregulation effect, one should also validate its use carefully in the system of choice since the concern has been raised that epigenetic alterations by epigenetic regulators such as Sir2a could spread to neighboring genomic loci and thereby complicate the interpretation of results (Walker and Lindner, 2019). Another option would be to test other endonuclease-dead Cas proteins, such as dCas12a, for their capacity to confer an efficient and specific knockdown in *P. knowlesi*. Cas12a, formerly known as Cpf1, also targets DNA but is smaller than Cas9, requires only a smaller sgRNA than Cas9 and leaves a staggered DSB instead of the blunt DSB left by Cas9 (Zetsche *et al.*, 2015). It might become especially interesting for *Plasmodium* research since its preferred PAM is -TTTV (V can be A, C, G) (Zetsche *et al.*, 2015) and is therefore uniquely applicable to the AT-rich genome of *Plasmodium* parasites (Gardner *et al.*, 2002). Thus, there are likely more possible sgRNAs per

target gene and the selection of these can be fine-tuned once definitive rules for efficient sgRNAs are elucidated. Irrespective of the chosen Cas protein, it would be a good addition to make its expression inducible. For example, Liang *et al.* raised concerns about constitutive expression of dCas9, including that the continuous downregulation of a gene could mask the true phenotype of the disruption through adaption of the parasite (Liang *et al.*, 2022). Another hypothetical side-effect they mentioned was that constant targeting of essential genes might be too deleterious which would also lead to important phenotypes to be missed.

The dCas9 in our system included a C-terminal *glmS* ribozyme that is activated by the addition of glucosamine to the growth medium, leading to self-cleavage of the ribozyme and thereby transcript instability and degradation (Prommana *et al.*, 2013; Briquet, Gissot and Silvie, 2022). However, it should be noted that the addition of glucosamine to control WT *P. falciparum* parasites showed an impact on their growth in a study performed in our lab (Wenz *et al.*, 2023). Another system allowing inducibility of expression that has been used in *Plasmodium* parasites is the TetR-DOZI-aptamer system. Here, the addition of a ligand displaces the TetR repressor from an aptamer sequence inserted at the 5' end of the transcript, thereby allowing the translation of the transcript (Briquet, Gissot and Silvie, 2022). Liang *et al.* have reported that they detected leaky expression of dCas9 using TetR-DOZI which is why they implemented the rapamycin-activable DiCre/*loxP* system for their study (Liang *et al.*, 2022) (see Fig. 1.11 C in chapter 1.6). In contrast to the *glmS* ribozyme or the TetR-DOZI-aptamer system, DiCre/*loxP* offers inducibility on the level of transcription: *loxP* sites are inserted flanking a region that is supposed to be removed or altered, in this case e.g. an open reading frame inhibiting the transcription of the gene of interest. The addition of rapamycin leads to the dimerization of DiCre which in turn cuts the DNA at the *loxP* sites and, depending on the orientation of the sites, either flips the flanked sequence or cuts it out. This DiCre approach has been reported to be leak-free and fast-acting (Liang *et al.*, 2022; Lucky *et al.*, 2024). Another fast-acting approach that has been used in *Plasmodium* is the knock-sideways system. Here, a protein of interest is fused to a FK506-binding protein (FKBP) domain and a FKBP-rapamycin binding (FRB) domain is fused to a targeting signal, together constituting the so-called “mislocalizer” (Kimmel *et al.*, 2023). Depending on the physiological localization of the protein of interest, e.g. the nucleus in the case of the dCas9, the targeting signal could be e.g. the parasite plasma membrane. In the presence of a rapalog, FKBP and FRB dimerize and the dCas9 would be transported to the parasite plasma membrane where it, thus, cannot interfere with transcription.

Concerning the functionality of the screen presented in this work, once the parasites entered exponential growth, the readout using RT-qPCR seemed to be able to monitor changes in relative construct abundance ratio well (Suppl. Fig. 9 and 10). By examining the raw Ct values, we found an excess of plasmid abundance during the first 14 and 20 cycles of the screen, respectively, that likely masked any dynamic changes in relative plasmid abundance before these timepoints. By treating the harvested samples with saponin and DNase I, I found out that the excess plasmid background seemed to neither originate from the medium or from uninfected RBCs that were present during transfection, nor stick to the surfaces, such as the tubes. Therefore, we concluded that the most likely source was the majority of segmented schizonts that did not survive the nucleofection but potentially still harbored plasmids. Although the growth medium was carefully exchanged every day for a week after transfection, the number of these dead segmenters decreased over time, but they were still present ca. 2 weeks after transfection. We reasoned that since these segmented parasites contain paramagnetic hemozoin crystals, one should be able to separate them following the recovery period in which surviving merozoites re-invade RBCs by magnetic-activated cell sorting. Unfortunately, not enough time remained to test this hypothesis.

In general, for the screening application that we used here, the readout via RT-qPCR worked very well. Since our library contained a sufficiently small number of 20 different constructs, I was able to design specific primers for each construct. As soon as the library size reaches a certain size, next-generation sequencing is often used as a readout. One recently published study that used Illumina-based sequencing as their readout was the previously mentioned small-scale CRISPRi screen in *P. falciparum* (Lucky *et al.*, 2024). Here, they used cultures that had previously been transfected separately with 9 different constructs targeting 9 genes. Expression of dCas9 fused to effector domains was inducible via DiCre/*loxP* so that they were able to mix parasites at equal numbers from each transfected cell line and then induce the knockdown at the same time to harvest samples at different timepoints after induction. From the samples, DNA was extracted and the sgRNA cassette including barcodes were PCR-amplified and prepared for Illumina-sequencing. This usually involves the ligation of specific adapters to the sequence of interest that allow it to be sequenced with Illumina technology. Lucky *et al.* expressed the thereby gained relative abundance of the plasmids as a fold-change and monitored this abundance over time for competitive growth, as well as growth in presence of antimalarial drugs (chloroquine and dihydroartemisinin) and growth under starvation and heat shock conditions. This way, they were able to identify how the up- or downregulation of the 9 genes affected the parasites' fitness under these conditions. Since their targets included the well-known proteins involved in drug

resistance, *P. falciparum* chloroquine resistance transporter and Kelch 13, they could validate their system by showing that dCas9-GCN5-mediated upregulation of these genes induced a fitness benefit when parasites were grown under chloroquine or dihydroartemisinin treatment, respectively. This study is intriguing and incorporated parts that we had planned to implement in our system, too, such as examining the effect targeted genes have on parasites' fitness when grown in the presence of antimalarial drugs or the easily scalable readout via next-generation sequencing. However, the approach that they used for most of the study, transfecting parasites with a single kind of construct each is not readily scalable and therefore inadequate for larger screens. In fact, the researchers show and briefly mentioned that they attempted transfection of plasmid pools of either 10 or 41 different constructs, respectively, but were faced with unequal construct coverage in the parasite population after transfection.

Another approach to advance the functional annotation of the *Plasmodium* genome was taken by Kimmel *et al.* who performed a gene-by-gene screen using the already mentioned knock-sideways method in *P. falciparum* (Kimmel *et al.*, 2023). They were able to tag all non-secretory proteins of unknown function encoded on chromosome 3, that had evidence of being expressed during the IDC, with a FKBP domain using their selection-linked-integration approach. Depending on their physiological localization they chose an appropriate signal target for each of the ca. 30 proteins. A clear advantage of this system is that one gains a lot of information per target, such as the cellular localization and (some) expression dynamics throughout the IDC under physiological and knockdown conditions. Additionally, further validation experiments can be performed more easily since the corresponding cell lines already exist. However, the scalability of the approach is also relatively limited.

3.3 Conclusion and open questions

This project, although it fell short of the initial expectations and hopes concerning *P. knowlesi* transfection efficiency and overall functionality of CRISPRi-mediated knockdown, constitutes a good starting point for further exploratory approaches to establish this powerful method in an experimental *Plasmodium* strain important for malaria research as described in chapters 3.1 and 3.2.

However, some open questions remain. This includes the multiplicity of vector uptake and the ensuing plasmid propagation in *P. knowlesi*. In the Carrasquilla *et al.* paper, the researchers showed that using *P. falciparum* schizont-stage parasite transfection 93% of cells took up only one plasmid while this fraction was only 54% and 51% when they used ring-stage parasites or

“pre-loading”, respectively (Carrasquilla *et al.*, 2020). Indeed, when I transfected both GFP- and mScarlet-expressing constructs as a pool in schizont-stage *P. knowlesi* parasites, I was only able to detect parasite cells that either expressed one or the other (Suppl. Fig. 2). However, these experiments should be repeated since the proportion of fluorescent cells was overall low (23.2%). While there is not much known about the propagation of plasmids in *P. knowlesi*, in *P. falciparum* it has been shown that episomally maintained plasmids that do not contain a centromeric sequence are unstable and segregated/propagated to daughter cells at random (van Dijk *et al.*, 1997; O'Donnell *et al.*, 2001).

In addition, the results obtained in this project might be reason to consider if future large-scale gene knockdown screens should be performed in *P. knowlesi* or if it is, in fact, possible to use *P. falciparum* to this end. The main advantages of *P. knowlesi* are the increased transfection efficiency, which is supported by the experiments shown in this thesis, and the more balanced GC-content of its genome, allowing a higher number of possible sgRNAs per target gene. In the hypothetical case that a dCas12a-based knockdown system could be established in *Plasmodium*, using *P. falciparum* would likely be the better choice due to the PAM -TTTV of (d)Cas12a.

Furthermore, using the *P. knowlesi* laboratory strain A1-H.1 also has some practical limitations, for example, its requirement for Duffy-positive blood. The blood that is used as a standard in our department is not tested for the Duffy-status and commercial kits usually do not specifically test for the heterozygous Fy^{null} allele phenotype. Therefore, in my experience, it has been difficult to predict the parasites' growth behavior as it could differ by e.g. 2-fold, depending on the Duffy phenotype of the blood used. This made preparing large amounts of synchronized parasites, as was required for *P. knowlesi* transfections and knockdown validation experiments challenging. Another technical difficulty I encountered is the IDC cycle length of ca. 27 h, since parasites had to be synchronized in 2-3 consecutive cycles to achieve a high level of synchrony in the culture, and then used for transfection. This meant that for several consecutive cycles the time of performing experiments shifted ca. 4-6 h each day (the longer *Plasmodium* parasites are kept out of their optimal growth environment in incubators, the slower their IDC cycle gets). Lastly, *P. knowlesi* is simply less well studied as *P. falciparum* and there are less information resources regarding its genome available, e.g. the annotation of its TSSs. However, if it becomes possible to use *P. knowlesi* in a high-throughput knockdown approach, the reported capacity of the A1-H.1 strain to still invade cynomolgus RBCs offers the possibility to perform an *in vivo* screen which would provide crucial information under physiological conditions of the *Plasmodium* infection.

Regarding *P. falciparum*'s poor transfection efficiency, as mentioned before, it had prevented large-scale/high-throughput approaches for a long time and dictated the use of massive amounts of parasites and resources in the case of the one saturation mutagenesis screen that has been performed (Zhang *et al.*, 2018) for which is, admittedly, very expensive and laborious. And although, it has not been possible to improve this strain's transfection efficiency much over the last ca. two decades, the screen by Zhang *et al.* demonstrated that it has been at least practically possible to target the whole *P. falciparum* genome, albeit with a fundamentally different system than CRISPR-Cas or CRISPRi.

Taken together, I believe both *Plasmodium* strains have clear advantages and disadvantages, so it might depend on the experimental and practical circumstances which strain will be used for further high-throughput genetic (knockdown) screens.

4. Materials and Methods

4.1 Materials

4.1.1 Cell lines

Parasite cell lines

Tab. 4.1: Parasite cell lines.

Parasite cell line	Characteristics	Source
<i>P. knowlesi</i> (<i>P.k.</i>) A1-H.1	Wildtype; parental line for all parasite cell lines in this thesis	Robert Moon, The London School of Hygiene & Tropical Medicine, London, UK
<i>P.k.</i> A1-H.1 WT + pPkconGFP	Transfected with episomal plasmid pPkconGFP	This thesis
<i>P.k.</i> A1-H.1-dCas9-p230p (<i>P.k.</i> A1-H.1-dCas9)	Constitutively expresses dCas9-3xHA; mixed (non-clonal)	Ann-Kathrin Mehnert, (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)
<i>P.k.</i> A1-H.1-dCas9 (B5)	Constitutively expresses dCas9-3xHA; cloned by limited dilution cloning (clone B5)	Ann-Kathrin Mehnert, (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)
<i>P.k.</i> A1-H.1-dCas9 (F6)	Constitutively expresses dCas9-3xHA; cloned by limited dilution cloning (clone B5)	Ann-Kathrin Mehnert, (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)
<i>P.k.</i> A1-H.1-dCas9 (B5) + pPkconGFP	Transfected with episomal plasmid pPkconGFP	This thesis
<i>P.k.</i> A1-H.1-dCas9 (B5) + pPkconmScarlet	Transfected with episomal plasmid pPkconmScarlet	This thesis
<i>P.k.</i> A1-H.1-dCas9 (B5) + pPkconGFP/pPkconmScarlet	Transfected with episomal plasmid pPkconGFP and pPkconmScarlet	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_VPS32_sgR1334 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_VPS32_sgR1334 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_VPS32_sgR1454 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_VPS32_sgR1454 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library (empty plasmid) using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_CEN4_sgR1128 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_CEN4_sgR1128 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_CEN4_sgR1529 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_CEN4_sgR1529 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_CEN4_sgR1922 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_CEN4_sgR1922 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library (empty plasmid) using nucleofector 2b	This thesis

<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_SAS6_sgR886 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_SAS6_sgR886 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_SAS6_sgR1185 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_SAS6_sgR1185 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_SAS6_sgR1298 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_SAS6_sgR1298 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_SAS6_sgR1472 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_SAS6_sgR1472 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library_SAS6_sgR1672 (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library_SAS6_sgR922 using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + pL6-opti-library (2b)	Constitutively expresses dCas9-3xHA; clone F6; transfected with episomal plasmid pL6-opti-library (empty plasmid) using nucleofector 2b	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + Mini screen library (n1) (4D Hi)	Transfected with library comprising 20 different sgRNA-expressing constructs (see Tab. 2.3); transfected with 4D nucleofector with 200 µg total plasmid DNA	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + Mini screen library (n1) (4D Lo)	Transfected with library comprising 20 different sgRNA-expressing constructs (see Tab. 2.3); transfected with 4D nucleofector with 20 µg total plasmid DNA	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + Mini screen library (n1) (2b Hi)	Transfected with library comprising 20 different sgRNA-expressing constructs (see Tab. 2.3); transfected with 2b nucleofector with 200 µg total plasmid DNA	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + Mini screen library (n1) (2b Lo)	Transfected with library comprising 20 different sgRNA-expressing constructs (see Tab. 2.3); transfected with 2b nucleofector with 20 µg total plasmid DNA	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + Mini screen library (n2) (4D)	Transfected with library comprising 20 different sgRNA-expressing constructs (see Tab. 2.3); transfected with 4D nucleofector with 20 µg total plasmid DNA	This thesis
<i>P.k.</i> A1-H.1-dCas9 (F6) + Mini screen library (n2) (2b)	Transfected with library comprising 20 different sgRNA-expressing constructs (see Tab. 2.3); transfected with 2b nucleofector with 20 µg total plasmid DNA	This thesis

Bacteria strain

Tab. 4.2: Bacteria strain.

Name	Description	Source	Product number
NEB 5-alpha F'Iq Competent <i>E. coli</i>	Chemically competent <i>E. coli</i> cells derived from DH5α	New England Biolabs (Ipswich, USA)	C2992

4.1.2 Equipment

Tab. 4.3: Equipment.

Equipment	Name	Company
Agarose gel electrophoresis	EV231	Consort (Turnhout, Belgium)
	E831	Consort (Turnhout, Belgium)
	UV-transilluminator Herolab UVT-28 L	Herolab (Wiesloch, Germany)
	Microwave 900&Grill	SEVERIN Elektrogeräte GmbH (Sundern, Germany)
Scales	Kern EW 600-2M	KERN & SOHN GmbH (Balingen, Germany)
	TE124S-0CE	Sartorius AG (Göttingen, Germany)
Cell culture	BVC professional vacuum pump	VACUUBRAND GMBH + CO KG (Wertheim, Germany)
	Neubauer counting chamber	neoLab Migge GmbH (Heidelberg, Germany)
	ENVAIReco safe Basic Plus Safety Cabinet	ENVAIR Deutschland GmbH (Emmendingen, Germany)
	Hera Safe KS 15	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
	Isotemp 210 Water Bath	Fisher Scientific International, Inc. (Hampton, New Hampshire, USA)
	Labo C201 Incubator	Labotect GmbH (Rosdorf, Germany)
	Sanyo CO2 Incubator MCO-17AI	Sanyo Electric Co., Ltd (Osaka, Japan)
Centrifuges	Heraeus Fresco 21 Centrifuge	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
	Heraeus Pico 21 Centrifuge	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
	Heraeus Multifuge 1 S-R	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
	Heraeus Multifuge 1.0 R	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
	Avanti J-26S XP with rotor JA-10	Beckman Coulter (Brea, California, USA)
	Mini centrifuge Su 1550	Sunlab GmbH (Aschaffenburg, Germany)
Nucleofactors	Nucleofector 2b Device	Lonza (Basel, Switzerland)
	4D Nucleofector Core Unit and X Unit	Lonza (Basel, Switzerland)
Refrigerators and freezers	Liebherr CN 3666	Liebherr-Hausgeräte GmbH (Ochsenhausen, Germany)
	Innova U725 Freezer (-80 °C)	New Brunswick Scientific Co., Inc. (Edison, New Jersey, USA)
	MediLine Freezer (-20 °C)	Liebherr-Hausgeräte GmbH (Ochsenhausen, Germany)
	MediLine Fridge (4 °C)	Liebherr-Hausgeräte GmbH (Ochsenhausen, Germany)
	Liebherr Comfort	Liebherr-Hausgeräte GmbH (Ochsenhausen, Germany)
Heating blocks	neoBlock 1 Heater	neoLab Migge GmbH (Heidelberg, Germany)
	ThermoMixer C	Eppendorf (Hamburg, Germany)
	AccuBlock™ Digital Dry Bath	Labnet International Inc. (Edison, New Jersey, USA)
Bacterial incubators	CH-4103	Infors AG (Bottmingen, Switzerland)
	IN75	Memmert (Schwabach, Germany)
Pipettes	Micropipette 0.2-2 µl	Gilson Incorporated (Middletown, Wisconsin, USA)
	Micropipette 0.5-10 µl	Sunlab GmbH (Aschaffenburg, Germany)
	Micropipette 2-20 µl	Gilson Incorporated (Middletown, Wisconsin, USA)

		Sunlab GmbH (Aschaffenburg, Germany)
	Micropipette 20-200 µl	Gilson Incorporated (Middelton, Wisconsin, USA)
	Micropipette 100-1000 µl	Gilson Incorporated (Middelton, Wisconsin, USA)
		Sunlab GmbH (Aschaffenburg, Germany)
	12 channel Multipipette 2-200 µl	Eppendorf (Hamburg, Germany)
Pipette aids	Sunlab digital pipetting aid	Sunlab GmbH (Aschaffenburg, Germany)
	Pipetus	Hirschmann Laborgeräte GmbH & Co. KG (Eberstadt, Germany)
	Accu-jet pro	Brand GMBH + CO. KG (Wertheim, Germany)
Real-Time-PCR System	QuantStudio 1	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Shakers and mixers	Orbital shaker HS 250 basic	IKA-Werke GmbH & CO. KG (Staufen, Germany)
	neoLabLine Vortex Mixer	neoLab Migge GmbH (Heidelberg, Germany)
Spectrophotometer	NanoPhotometer NP80	Implen (München, Germany)
Thermal cyclers	FlexCycler2	Analytik Jena (Jena, Germany)
Microscopes	Leica DM IL	Leica Microsystems GmbH (Wetzlar, Germany)
	Keyence BZ-X800	Keyence Corporation (Osaka, Japan)
	Leica SP8 TCS DLS	Leica Microsystems GmbH (Wetzlar, Germany)
Flow cytometers	BD FACSCanto II	BD Bioscience (Franklin Lakes, New Jersey, USA)
	BD FACSCelesta	BD Bioscience (Franklin Lakes, New Jersey, USA)
Miscellaneous	Fume Hood	Wesemann GmbH (Syke, Germany)
	Ice Dispenser AF 80	Scotsman Ice Systems (Vernon Hills, Illinois, USA)
	Heating plate and magnetic stirrer RH basic 2	IKA-Werke GmbH & CO. KG (Staufen, Germany)

4.1.3 Consumables and reagents

Consumables

Tab. 4.4: Consumables.

Product name	Company
Cell culture dishes (10 cm, 20 cm)	Greiner Bio-One GmbH (Frickenhausen, Germany)
Cell culture plates (6-well, 24-well, 96-well)	Greiner Bio-One GmbH (Frickenhausen, Germany)
Cryogenic vials (2 ml)	Greiner Bio-One GmbH (Frickenhausen, Germany)
Falcon tubes (15 ml, 50 ml)	Greiner Bio-One GmbH (Frickenhausen, Germany)
Filter tips (10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Sorenson Bioscience Inc. (Salt Lake City, Utah, USA)
Ibidi microscopy dishes (8-well ibidi treat)	Ibidi (Fitchburg, Wisconsin, USA)
Parafilm M	Bemis (Neenah, Wisconsin, USA)
PCR tubes (single tubes, 8-strips)	Biozym Scientific GmbH (Hessisch Oldendorf, Germany)
Petri dishes	Greiner Bio-One GmbH (Frickenhausen, Germany)
Pipette tips (unfiltered) (10 µl, 20 µl, 200 µl, 1000 µl)	Steinbrenner Laborsysteme GmbH (Wiesenbach, Germany)
Precision wipes (KIM wipes)	Kimberley-Clark Professional (Irving, Texas, USA)
Protein LoBind Tubes (1.5 ml)	Eppendorf AG (Hamburg, Germany)
Reaction tubes (1.5 ml, 2 ml)	SARSTEDT AG & Co. KG (Nümbrecht, Germany)
Serological pipettes (5 ml, 10 ml, 25 ml, 50 ml)	Greiner Bio-One GmbH (Frickenhausen, Germany)

MicroAmp optical 96-well reaction plate	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
MicroAmp optical adhesive film	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)

Chemical reagents

Tab. 4.5: Chemical reagents.

Product name	Company
2-Mercaptoethanol	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Acetone (anhydrous)	Sigma-Aldrich, Inc. (St. Louis, USA)
Agarose NEEO ultra-quality EEO = 0,05-0,13	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Albumax II (11021045)	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Albumin (BSA)	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Concanavalin A (C2010)	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
Concentrated erythrocytes (O+)	DRK Blutbank Mannheim (Mannheim, Germany)
CutSmart buffer	New England Biolabs (Ipswich, Massachusetts, USA)
Dimethyl sulfoxide (DMSO)	Honeywell (Seelze, Germany)
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco by life technologies (Paisley, UK)
dNTP mix (10 mM) (N0447L)	New England Biolabs (Ipswich, Massachusetts, USA)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
Ethanol absolute	VWR Chemicals (Radnor, Pennsylvania, USA)
Ethanol (96%, 99.9%)	Zentralbereich Neuenheimer Feld (Heidelberg, Germany)
Gentamycin sulphate solution (HN09.1)	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Glutaraldehyde solution, grade I, 25% in H ₂ O	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
Glycerol	AppliChem GmbH (Darmstadt, Germany)
Hemacolor Rapid staining of blood smear Solution	VWR Chemicals (Radnor, Pennsylvania, USA)
Hemacolor Rapid staining of blood smear Solution 3	VWR Chemicals (Radnor, Pennsylvania, USA)
HEPES (H4034)	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
Horse serum	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Hypoxanthine (Z-41-M)	c.c.pro GmbH (Oberlora, Germany)
Isopropanol	Zentralbereich Neuenheimer Feld (Heidelberg, Germany)
LB Broth (Lennox)	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Methanol	Zentralbereich Neuenheimer Feld (Heidelberg, Germany)
Nycodenz (1002424)	Axis-Shield (Dundee, UK)
Paraformaldehyde (PFA) 16%	Electron Microscopy Sciences (Hatfield, Pennsylvania, USA)
Phosphate-buffered saline (PBS)	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
Phusion polymerase HF reaction buffer (5x)	New England Biolabs (Ipswich, Massachusetts, USA)
Purple loading dye	New England Biolabs (Ipswich, Massachusetts, USA)
RPMI 1640 GlutaMAX, phenol-red (61870010)	Gibco by life technologies (Paisley, UK)
Saponin Quillaja sp.	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
SOC outgrowth medium	New England Biolabs (Ipswich, Massachusetts, USA)
Sodium acetate	Merck KGaA (Darmstadt, Germany)
Sodium borohydride (NaBH ₄)	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
Sodium chloride (NaCl)	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
SYBR Green I	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
T4 DNA ligase buffer (10x)	New England Biolabs (Ipswich, Massachusetts, USA)
TRIS	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)

Triton-X-100	Merck KGaA (Darmstadt, Germany)
Tween-20	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
WR99210	Jacobus Pharmaceuticals (Plainsboro Township, New Jersey, USA)

4.1.4 Buffers, media and solutions

Tab. 4.6: Buffers, media and solutions.

Name	Composition	Purpose
100% Nycodenz solution	27.6 g Nycodenz powder	Parasite synchronization
	10 mM TRIS	
	ddH ₂ O to 100 ml (pH=7)	
Complete cell culture medium (cRPMI)	RPMI 1640, GlutaMAX, phenol-red (410 ml)	Standard parasite culture
	25 mM HEPES	
	12.5 µg/ml Gentamycin sulphate solution	
	0.2 mM Hypoxanthine	
	0.5% Albumax II	
	10% Horse serum	
Freezing solution	28% Glycerol	Freezing of culture cryo stocks
	3% D-Sorbitol	
	0.65% NaCl	
Incomplete cell culture medium (iRPMI)	RPMI 1640, GlutaMAX, phenol-red (500 ml)	Cell culture (washing)
	25 mM HEPES	
	12.5 µg/ml Gentamycin sulphate solution	
TE buffer, pH 7.5	10 mM TRIS -HCl, pH 7.5	Transfection (DNA resuspension)
	1 mM EDTA	

4.1.5 Antibodies, dyes and DNA markers

Antibodies

Tab. 4.7: Antibodies.

Name	Species	Application	Dilution	Supplier
α -Tubulin, B-5-1-2, T5168, monoclonal	Mouse	IFA	1:500	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)
α -GFP, monoclonal	Rabbit	IFA	1:50	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
α -HA clone 3F10	Rat	IFA	1:500	Roche (Basel, Switzerland)
α -mouse-Atto-647	Goat	IFA	1:1000	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
α -rabbit Alexa- Fluor Plus 488		IFA	1:1000	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
α -rat Alexa- Fluor 568	Goat	IFA	1:1000	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)

Dyes

Tab. 4.8: Dyes.

Name	Application	Dilution	Product Number	Source/supplier
Hoechst 33342	IFA	1:10 000	H3570	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
SYBR Green I	Flow cytometry	1:10 000	S7563	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)
DRAQ5	Flow cytometry	1:1000	DR05500	BioStatus (Shepshed, UK)
Midori Green Advance	Agarose gel electrophoresis	1:500	MG04	Nippon Genetics Europe (Düren, Germany)

DNA size markers

Tab. 4.9: DNA size markers.

Name	Company	Product no.
1 kb DNA Ladder	New England Biolabs (Ipswich, Massachusetts, USA)	N3232
100 bp DNA Ladder	New England Biolabs (Ipswich, Massachusetts, USA)	N3231

4.1.6 Enzymes and kits

Enzymes

Tab. 4.10: Enzymes.

Name	Purpose	Company	Product number
AvrII	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R0174
BamHI-HF	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R3136L
BbsI-HF	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R3539
BtgZI	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R0703S
EcoRI-HF	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R3101
NotI-HF	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R3189
SacI-HF	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	R3156S
DNase I	Sampling (screen)	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)	AM2239
Phusion Polymerase	Cloning, PCR	New England Biolabs (Ipswich, Massachusetts, USA)	M0530
T4 Ligase	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	M0202
Taq Polymerase	Cloning, PCR	New England Biolabs (Ipswich, Massachusetts, USA)	M0273
Hifi Assembly Master Mix	Cloning	New England Biolabs (Ipswich, Massachusetts, USA)	E2621
SsoAdvanced Universal SYBR Green Supermix	RT-qPCR	BioRad (Hercules, California, USA)	1725271

Commercial kits

Tab. 4.11: Commercial kits.

Name	Company	Product number
GenElute HP Plasmid Miniprep Kit	Sigma-Aldrich, Inc. (St. Louis, Missouri, USA)	NA0150
NucleoBond Xtra Midi Plus	Macherey-Nagel (Düren, Germany)	740412.50
High Pure PCR Product Purification Kit	Roche (Mannheim, Germany)	11732676001
DNeasy Blood & Tissue Kit (50)	Qiagen (Hilden, Germany)	69504
RNeasy Mini Kit	Qiagen (Hilden, Germany)	74104
TURBO DNA-free Kit	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)	AM1907
First Strand cDNA Synthesis Kit	Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA)	K1612
Basic Parasite Nucleofector Kit 1	Lonza (Basel, Switzerland)	VMI-1011
P3 Primary Cell 4D X Kit L	Lonza (Basel, Switzerland)	V4XP-3024

4.1.7 Plasmids

All plasmids that were used or generated by Ann-Kathrin Mehnert for the generation of the *P. knowlesi* A1-H.1-dCas9-p230p line are listed in her Master thesis (A.-K. Mehnert, Master thesis, 2019).

Tab. 4.12: Plasmids.

Name	Description	Source
pPkconGFP	Constitutive episomal expression of eGFP through PkHSP70 promoter; was used as reporter construct	Robert Moon, The London School of Hygiene & Tropical Medicine, London, UK
pCas9/no_guide	Cas9 expression plasmid; was used as template to amplify <i>P. knowlesi</i> -specific U6 5' UTR	Robert Moon, The London School of Hygiene & Tropical Medicine, London, UK
pL6-opti-library	Library plasmid for sgRNA expression; contains an optimized tracrRNA sequence; sgRNAs can be cloned in via BtgZI sites	Master thesis Ann-Kathrin Mehnert, (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)
pL6-opti-library_BbsI-in-hDHFR-removed_1	Based on pL6-opti-library; BbsI site in the hDHFR cassette removed by amplifying the corresponding sequence from pCas9-sg_1xBbsI	This thesis
pL6-opti-library_BbsI	Based on pL6-opti-library_BbsI-in-hDHFR-removed_1 plasmid; BtgZI restriction sites were exchanged to BbsI restriction sites by amplifying the corresponding sequence from pCas9-sg_1xBbsI	This thesis
pCas9-sg_1xBbsI	Cas9 expression plasmid; was based on pCas9/no_guide where BtgZI sites were exchanged with BbsI sites	Yannik Voß (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)

pPkconmScarlet	Based on pPkconGFP; constitutive episomal expression of mScarlet through PkHSP70 promoter; was used as reporter construct	This thesis
pL6-opti-Vps32gR454	pL6-opti-library plasmid that contains sgRNA Vps32gR454 (note, according to updated nomenclature this is sgRNA VPS32 sgR 1454) in sgRNA expression cassette	Master thesis Ann-Kathrin Mehnert, (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)
pL6-opti-Vps32gR334 (negative)	pL6-opti-library plasmid that contains sgRNA Vps32gR334 (note, according to updated nomenclature this is sgRNA VPS32 sgR 1334) in sgRNA expression cassette; was used as negative knockdown control	Master thesis Ann-Kathrin Mehnert, (Center for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany)
pL6-opti-library_Vps32_sgR442	pL6-opti-library plasmid that contains sgRNA Vps32 sgR442 (note, according to updated nomenclature this is sgRNA VPS32 sgR 1442) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen4gR128	pL6-opti-library plasmid that contains sgRNA Cen4 sgR128 (note, according to updated nomenclature this is sgRNA CEN4 sgR 1128) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen4gR529	pL6-opti-library plasmid that contains sgRNA Cen4 sgR529 (note, according to updated nomenclature this is sgRNA CEN4 sgR 1529) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen4gR922	pL6-opti-library plasmid that contains sgRNA Cen4 sgR922 (note, according to updated nomenclature this is sgRNA CEN4 sgR 1922) in sgRNA expression cassette	This thesis
pL6-opti-library_Sas6sgR886	pL6-opti-library plasmid that contains sgRNA Sas6 sgR886 (note, according to updated nomenclature this is sgRNA SAS6 sgR 886) in sgRNA expression cassette	This thesis
pL6-opti-library_Sas6sgR1185	pL6-opti-library plasmid that contains sgRNA Sas6 sgR1185 (note, according to updated nomenclature this is sgRNA SAS6 sgR 1185) in sgRNA expression cassette	This thesis
pL6-opti-library_Sas6sgR1298	pL6-opti-library plasmid that contains sgRNA Sas6 sgR1298 (note, according to updated nomenclature this is sgRNA SAS6 sgR 1298) in sgRNA expression cassette	This thesis
pL6-opti-library_Sas6sgR1472	pL6-opti-library plasmid that contains sgRNA Sas6 sgR1472 (note, according to updated nomenclature this is sgRNA SAS6 sgR 1472) in sgRNA expression cassette	This thesis
pL6-opti-library_Sas6sgR1672	pL6-opti-library plasmid that contains sgRNA Sas6 sgR1672 (note, according to updated nomenclature this is sgRNA SAS6 sgR 1672) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen3sgR1550	pL6-opti-library plasmid that contains sgRNA Cen3 sgR1550 (note, according to updated nomenclature this is sgRNA CEN3 sgR 1550) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen3sgR1566	pL6-opti-library plasmid that contains sgRNA Cen3 sgR1566 (note, according to updated nomenclature this is sgRNA CEN3 sgR 1566) in sgRNA expression cassette	This thesis

pL6-opti-library_Cen3sgR1681	pL6-opti-library plasmid that contains sgRNA Cen3 sgR1681 (note, according to updated nomenclature this is sgRNA CEN3 sgR 1681) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen3sgR1729	pL6-opti-library plasmid that contains sgRNA Cen3 sgR1729 (note, according to updated nomenclature this is sgRNA CEN3 sgR 1729) in sgRNA expression cassette	This thesis
pL6-opti-library_Cen3sgR1803	pL6-opti-library plasmid that contains sgRNA Cen3 sgR1803 (note, according to updated nomenclature this is sgRNA CEN3 sgR 1803) in sgRNA expression cassette	This thesis

4.1.8 Primers and oligonucleotides

Primers

Tab. 4.13: Primers.

No.	Primer ID	Primer sequence (5'→3')	Template	Application
1	Vps32sgR334_F	GTTTATACCTGGATTAgtttCagagc	Plasmid	RT-qPCR
2	Vps32sgR442_F	attTGTGACAAATTGCG	Plasmid	RT-qPCR
3	Vps32sgR454_F	GCAACTTGGTTCCTCCCTC	Plasmid	RT-qPCR
4	Cen4sgR128_F	GCACGTCATCACCTCgtttc	Plasmid	RT-qPCR
5	Cen4sgR529_F	CACAAATGTGTCACAAAGGgtttc	Plasmid	RT-qPCR
6	Cen4sgR922_F	GCTGAATGCTTAGCAAACGg	Plasmid	RT-qPCR
7	Sas6sgR886_F	AAGGAGGAGCCCCGC	Plasmid	RT-qPCR
8	Sas6sgR1185_F	CGAAACTGCCGCACG	Plasmid	RT-qPCR
9	Sas6sgR1298_F	GGTGAGATTCTCCTTCAAGCg	Plasmid	RT-qPCR
10	Sas6sgR1472_F	CACGAAAACCTCCACAGGg	Plasmid	RT-qPCR
11	Sas6sgR1672_F	CGGTAGACGGAGCACTGgtt	Plasmid	RT-qPCR
12	Cen3sgR1550_F	GAGTGGGAAGCCTTACCG	Plasmid	RT-qPCR
13	Cen3sgR1566_F	CCGTGGTGCGATCAGCCCA	Plasmid	RT-qPCR
14	Cen3sgR1681_F	GATATACTGCTCGTGCGTAAg	Plasmid	RT-qPCR
15	Cen3sgR1729_F	CAAGAAGTCACTAATTCGAGg	Plasmid	RT-qPCR
16	Cen3sgR1782_F	GGACTGGGGAGATAAAGAAG	Plasmid	RT-qPCR
17	Cen3sgR1798_F	AGCTCGTGATGCGGACgtt	Plasmid	RT-qPCR
18	Cen3sgR1803_F	AAGTAAAGAGCTCGTGATGg	Plasmid	RT-qPCR
19	Cen3sgR1919_F	attGCTCGAAAGCGTGCA	Plasmid	RT-qPCR
20	pL6_empty_F	GGGTCTTCgaGAAGACCTgtttc	Plasmid	RT-qPCR
21	hDHFR_1_F	GGTCTGGATAGTTGGTGGCA	Plasmid	RT-qPCR
22	hDHFR_1_R	GCCTTTCTCCTCCTGGACAT	Plasmid	RT-qPCR
23	hDHFR_2_F	TGGTTGGTTCGCTAAACTGC	Plasmid	RT-qPCR
24	hDHFR_2_R	AATTCGTTCTGAGCGGTGG	Plasmid	RT-qPCR
25	PKNH_Vps32_1_F	GGAGGAGATAAACCAGGCCC	cDNA	RT-qPCR
26	PKNH_Vps32_1_R	AGCTGTTCTCTGGCCTTCAC	cDNA	RT-qPCR
27	PKNH_Cen4_1_F	GCGCTCTTCGACCCTAATGA	cDNA	RT-qPCR
28	PKNH_Cen4_1_R	TCACTGAGCGGGTCGTTT	cDNA	RT-qPCR
29	PKA1H_Sas6_1_F	CTTGATGGGGAAAGGGGCAT	cDNA	RT-qPCR

30	PKA1H_Sas6_1_R	TCCCGTTCGGGTGTGTTACTG	cDNA	RT-qPCR
31	PKNH_ Serine- tRNA-ligase_1_F	CCAGAACGACGAGCTAAGCA	cDNA	RT-qPCR
32	PKNH_ Serine- tRNA-ligase_1_R	ATGTGCTCCAGCTTCCTTCC	cDNA	RT-qPCR
33	pL6_opti_library_Bb sI_R	gactctggaagaaaatgagc	Plasmid	Cloning
34	pL6_opti_library_Bb sI_F	Aatttaattttattcgaaaggatccatgggtgctgctaaactg	Plasmid	Cloning
35	pL6_opti_lib_BbsI_ exchange	ttcatattaagtataataAGGTCTTCcGAAGACCCgtttccagcat agctctg	Plasmid	Cloning
36	pL6_opti_lib_BbsI_ ex_revcom	cagagctatgctggaacGGGTCTTCgaGAAGACCTttatatactt aatatgaa	Plasmid	Cloning
37	pL6-library_seqR	cgactcgggtccacttttc	Plasmid	Sequencing
38	pL6-library_seqF	tggatttctacacatcttgaggtt	Plasmid	Sequencing

Oligonucleotides

Tab. 4.14: Oligonucleotides.

sgRNA ID	Oligo ID	Strand orientation	Oligo sequence (5'→3')
VPS32 sgR 1334	Vps32_neg_gR33 4-F	Sense	catattaagtatataatattTTTGTTTATACCTGGATTtAgtttcagagctatgctg gaa
	Vps32_neg_gR33 4-R	Antisense	ttccagcatagctctgaaacTAAATCCAGGTATAAAACAAAaatattatatacttaa tatg
VPS32 sgR 1442	Vps32_sgR442_S	Sense	catattaagtatataatattTTGTGACAATTTGCGCAACTgtttcagagctatgctg gaa
	Vps32_sgR442_A	Antisense	ttccagcatagctctgaaacAGTTGCGCAAAATGTGCACAAaatattatatacttaa tatg
VPS32 sgR 1454	Vps32_gR454-F	Sense	catattaagtatataatattGCGCAACTTGGTTCCCCCTCgtttcagagctatgctg gaa
	Vps32_gR454-R	Antisense	ttccagcatagctctgaaacGAGGGGGAACCAAGTTGCGCAaatattatatactt aatatg
CEN4 sgR 1128	Cen4_gR128_S	Sense	catattaagtatataatattTGGGGGCACGTCATCACCTCgtttcagagctatgct ggaa
	Cen4_gR128_A	Antisense	ttccagcatagctctgaaacGAGGTGATGACGTGCCCCCAaatattatatactta atatg
CEN4 sgR 1529	Cen4_gR529_S	Sense	catattaagtatataatattGCACAAATGTGTACACAAAGGgtttcagagctatgct ggaa
	Cen4_gR529_A	Antisense	ttccagcatagctctgaaacCCTTTGTGACACATTTGTGCAaatattatatactta atg
CEN4 sgR 1922	Cen4_gR922_S	Sense	catattaagtatataatattTGCTGAATGCTTAGCAAACGgtttcagagctatgct ggaa
	Cen4_gR922_A	Antisense	ttccagcatagctctgaaacCGTTTGCTAAGCATTCAGCAaatattatatactta tatg
SAS6 sgR 886	Sas6_sgR886_S	Sense	catattaagtatataatattAAGGAGGAGCCCCGCGGAAAggtttcagagctatgc tggaa
	Sas6_sgR886_A	Antisense	ttccagcatagctctgaaacTTTCCGCGGGGCTCCTCCTTaatattatatactta atg
SAS6 sgR 1185	Sas6_sgR1185_S	Sense	catattaagtatataatattAGCGCGCGAAACTGCCGCACgtttcagagctatgct ggaa
	Sas6_sgR1185_A	Antisense	ttccagcatagctctgaaacGTGCGGCAGTTTCGCGCGCTaatattatatactta atatg
SAS6 sgR 1298	Sas6_sgR1298_S	Sense	catattaagtatataatattGGTGAGATTCTCCTTCAAGCgtttcagagctatgctg gaa

	Sas6_sgR1298_A	Antisense	ttccagcatagctctgaaacGCTTGAAGGAGAATCTCACCAatattataacttaa tatg
SAS6 sgR 1472	Sas6_sgR1472_S	Sense	catattaagtataataattTTCACGAAAACCTCCACAGGgtttcagagctatgct ggaa
	Sas6_sgR1472_A	Antisense	ttccagcatagctctgaaacCCTGTGGAGTTTTTCGTGCAAatattataacttaa tatg
SAS6 sgR 1672	Sas6_sgR1672_S	Sense	catattaagtataataattACGCGGTAGACGAGCACTGgtttcagagctatgct tggaa
	Sas6_sgR1672_A	Antisense	ttccagcatagctctgaaacCAGTGCTCCGTCTACCGCGTaatattataacttaa tatg
CEN3 sgR 1550	Cen3_sgR1550_S	Sense	catattaagtataataattCCGAGTGGGAAGCCTTACCGgtttcagagctatgct ggaa
	Cen3_sgR1550_A	Antisense	ttccagcatagctctgaaacCGGTAAGGCTTCCCACTCGGaatattataacttaa tatg
CEN3 sgR 1566	Cen3_sgR1566_S	Sense	catattaagtataataattACCGTGGTGCATCAGCCCAgtttcagagctatgct ggaa
	Cen3_sgR1566_A	Antisense	ttccagcatagctctgaaacTGGGCTGATCGCACACGGTaatattataacttaa atatg
CEN3 sgR 1681	Cen3_sgR1681_S	Sense	catattaagtataataattGATATACTGCTCGTGCCTAAgtttcagagctatgct ggaa
	Cen3_sgR1681_A	Antisense	ttccagcatagctctgaaacTTACGCACGAGCAGTATATCaatattataacttaa tatg
CEN3 sgR 1729	Cen3_sgR1729_S	Sense	catattaagtataataattCAAGAAGTCACTAATTCGAGgtttcagagctatgct ggaa
	Cen3_sgR1729_A	Antisense	ttccagcatagctctgaaacCTCGAATTAGTGACTTCTTGaatattataacttaa atg
CEN3 sgR 1782	Cen3_sgR1782_S	Sense	catattaagtataataattGGACTGGGGAGATAAAGAAGgtttcagagctatg ctggaa
	Cen3_sgR1782_A	Antisense	ttccagcatagctctgaaacCTTCTTTATCTCCCCAGTCCAatattataacttaata tg
CEN3 sgR 1798	Cen3_sgR1798_S	Sense	catattaagtataataattAAAGAGCTCGTGATGCGGACgtttcagagctatgct ggaa
	Cen3_sgR1798_A	Antisense	ttccagcatagctctgaaacGTCCGCATCACGAGCTCTTTaatattataacttaata atg
CEN3 sgR 1803	Cen3_sgR1803_S	Sense	catattaagtataataattAAAGTAAAGAGCTCGTGATGgtttcagagctatgct ggaa
	Cen3_sgR1803_A	Antisense	ttccagcatagctctgaaacCATCACGAGCTCTTTACTTTaatattataacttaata atg
CEN3 sgR 1919	Cen3_sgR1919_S	Sense	catattaagtataataattGCTCGAAAGCGTGACACACgtttcagagctatgct ggaa
	Cen3_sgR1919_A	Antisense	ttccagcatagctctgaaacGTGTGTGCACGCTTTCGAGCaatattataactta atatg

4.1.9 Software, tools and online resources

Tab. 4.15: Software, tools and online resources.

Name	Description	Source/reference
ApE	A freely available program to view and manipulate DNA <i>in silico</i>	M. Wayne Davis, (Davis and Jorgensen, 2022)
Fiji	Open-source image processing package based on the ImageJ2 distribution of ImageJ	(Schindelin <i>et al.</i> , 2012)
FlowJo	Platform for single-cell flow cytometry analysis	BD Bioscience (Franklin Lakes, New Jersey, USA)
Graphpad Prism	2D graphing and statistics software	GraphPad Software (San Diego, California USA)

Integrative Viewer	Genome	Open-source software; interactive tool to visualize genomics data	(Robinson <i>et al.</i> , 2011); https://igv.org/
SnapGene Viewer		Free version of SnapGene molecular biology software used to visualize and annotate plasmids and DNA sequences	SnapGene (Chicago, Illinois, USA)
QuantStudio Analysis Software	Design	Software to set up RT-qPCR experiments and analyze resulting data	Applied Biosystems (Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA))
Cufflinks		Free, open-source software tool; was used to estimate transcript levels from RNA-seq reads	(Trapnell <i>et al.</i> , 2012)
StringTie		Free, open-source software tool; was used to assemble all RNA-seq reads upstream of genes from the <i>P. knowlesi</i> H genome	(Pertea <i>et al.</i> , 2015)
DREME		Motif-based sequence analysis tool; was used to identify recurring motifs in sequences flanking TSSs	(Bailey, 2011); https://meme-suite.org/meme/doc/overview.html
MEME		Motif-based sequence analysis tool; was used to identify recurring motifs in sequences flanking TSSs	(Bailey <i>et al.</i> , 2009); https://meme-suite.org/meme/doc/overview.html
FIMO		Motif-based sequence analysis tool; was used to map identified motifs to the <i>P. knowlesi</i> H genome	(Grant, Bailey and Noble, 2011) ; https://meme-suite.org/meme/doc/overview.html
EuPaGDT		Online software tool to design and rate sgRNA sequences specific to eukaryotic pathogens	(Peng and Tarleton, 2015) ; http://grna.ctegd.uga.edu/
PlasmoDB		Online Plasmodium informatics resource; part of VEuPathDB	(Alvarez-Jarreta <i>et al.</i> , 2024) ; https://plasmodb.org/plasmo/app/

4.2 Methods

4.2.1 Parasite culture

Unless stated otherwise, medium and solutions that came into contact with parasites were prewarmed to 37°C.

4.2.1.1 Standard parasite culture

P. knowlesi parasites were cultured in human O⁺ RBCs in complete RPMI (cRPMI) at a hematocrit of 2.5% using cell culture dishes (10 or 20 cm) or 6-well plates. The parasites were kept in incubators with 90% humidity, 5% O₂ and 3% CO₂. Parasitemia was checked 2-3 times a week by thin blood smear (dried, fixed with methanol and stained with Hemacolor red and blue dyes) under a brightfield tabletop microscope (Nikon E100). For standard culture, parasitemia was maintained below 10% by splitting.

4.2.1.2 Freezing and thawing of parasite cryostocks

Since ring-stage parasites are the only stages that survive the freezing and thawing processes, cultures were allowed to reach >1% of ring-stage parasites before freezing. *P. knowlesi* parasites in 1 ml infected (iRBCs) were pelleted in a 50 ml tube at 800g for 5 min, the supernatant removed, and freezing solution was added dropwise and sequentially: 400 µl, 1.2 ml, 2.4 ml, with 5 min incubation at room temperature between each step. Parasites in the freezing solution was divided into 4x cryo vials and kept at -80°C.

For thawing parasites, cryo vials were retrieved from the -80°C ultra-low temperature freezer, the content allowed to defrost and transferred into a 50 ml tube. While shaking the tube, 200 µl 12% NaCl solution was added dropwise to the parasite solution and incubated for 5 min incubation at room temperature. Then, 9 ml 1.6% NaCl solution was added dropwise while shaking the tube. The solution was pelleted at 800g for 5 min and the supernatant removed. The RBC pellet was resuspended in 10 ml fresh cRPMI, transferred to a 10 ml cell culture dish, 250 µl fresh RBCs were added and parasites were placed in the incubator.

4.2.1.3 Parasite synchronization with Nycodenz gradient centrifugation

P. knowlesi parasites were synchronized using a Nycodenz gradient centrifugation. For this, a 55% Nycodenz solution was prepared fresh from the 100% Nycodenz stock with incomplete RPMI (iRPMI). 1-2 ml iRBCs were pelleted in a 50 ml tube at 800g for 5 min, resuspended in 25-35 ml iRPMI and underlaid with 10 ml 55% Nycodenz. For this, a glass Pasteur pipette was inserted into the tube and the Nycodenz solution was carefully pipetted into the Pasteur pipette so that it created a layer underneath the parasite solution (the separation between the two liquids should be as intact as possible). The tube was then centrifuged at 1500g, with a level 2 acceleration and no brake for 12 min. After centrifugation, the supernatant was removed a bit so that the brown schizont-layer could be carefully aspirated using a 1000 µl micropipette. The schizont-layer was transferred to a new 50 ml tube, diluted with 2x volume of iRPMI and pelleted at 800g for 5 min. The supernatant was removed, the schizont-containing pellet was resuspended in an appropriate amount of cRPMI and RBCs (depended on the percentage of schizont-stage parasites in the initial culture) and the parasites were placed in the incubator.

If ring- and trophozoite-stage parasites are desired, the iRBC-pellet after centrifugation can be washed with iRPMI, resuspended in cRPMI and placed in the incubator as described above.

4.2.1.4 Counting parasites in a Neubauer chamber

Parasites were diluted 1:25 or 1:50 in iRPMI and the Neubauer chamber was prepared by cleaning it with ethanol and drying it with lint-free wipes. The cover slip was attached to the chamber so that Newton rings were visible. 10 µl of diluted parasite solution was carefully pipetted into the chamber and parasites were counted using a tabletop brightfield microscope (Nikon E100) with 40x objective and phase contrast. The number of parasites was counted in the 4 big squares of the chamber, their average was determined and the number of total parasites calculated by

$$\frac{\text{Number of parasites}}{\text{ml}} = \text{Average number of parasites} \times 10^4 \times \text{dilution factor}$$

4.2.1.5 Plasmid precipitation for transfection

Plasmid was produced by inoculating a 200 ml LB culture from bacterial glycerol stocks stored at -80°C for overnight growth and performing a Midiprep the following day according to manufacturer's instructions. In general, 20 µg plasmid were precipitated per transfection reaction. In case of two plasmids co-precipitating, 20 µg of each plasmid was precipitated together. For the screen, in all reactions except the "4D Hi" and "2b Hi" reactions of the first replicate, 20 µg total plasmid containing all 20 constructs were precipitated together. In the "4D Hi" and "2b Hi" reactions, 200 µg total plasmid was precipitated.

Plasmid DNA (solved in Midi elution buffer) was mixed with 1/10 volume of 3 M sodium acetate (pH=5.3) and 2 volumes of cold ethanol absolute and incubated at -20°C for 1h or overnight. Precipitated DNA was pelleted at 16 000g for 15 min at 4°C, washed with cold 70% ethanol and centrifuged again with the same settings. The DNA pellet was air-dried in the laminar flow hood to keep it sterile and dissolved in 10 µl sterile TE buffer by aspirating up and down with a micropipette. Plasmid DNA was stored at -20°C until use.

4.2.1.6 Parasite transfection

For transfecting *P. knowlesi* parasites at the segmented-schizont stage, the parasites had to be tightly synchronized. For transfections with highest efficiencies, the first Nycodenz synchronization was performed as described above with cultures of 1-2 ml iRBCs at a parasitemia of >5% 3 cycles before the transfection. Schizont-stage parasites were transferred into an appropriate volume of cRPMI and fresh RBCs, depending on the yield of synchronized parasites. The second synchronization was performed ca. 28-30 h later to allow egress of the earliest schizonts and thereby tightening the synchronization. Again 28-30 h later, another

synchronization was performed and the harvested schizont-stage parasites were allowed to re-invade fresh RBCs for 3 h. For this, schizont-stage parasites were resuspended in medium with 5% hematocrit (e.g., 2 ml RBCs in 30 ml cRPMI) and placed on an orbital shaker at 80 rpm at 37°C to maximize invasion and minimize the occurrence of double infections. Afterwards, these ring-stage parasites were harvested with the same synchronization technique but the pellet containing ring-stage parasites was kept instead of the schizont-layer.

Schizont-stage parasites were then harvested 27-28 h later with another Nycodenz gradient centrifugation, resuspended in cRPMI (e.g. 2 ml for 5×10^8 parasites) and treated with the egress inhibitor ML10 at a final concentration of 150 nM at 37°C for 2 h. During this incubation period, the number of schizont-stage parasites was counted in a Neubauer chamber as described above. After the ML10 treatment, schizont-stage parasites were washed 3x with 2 ml iRPMI (pelleted at 400g for 2 min in between), resuspended in 2 ml cRPMI and incubated at 37°C for 15 min. In the meantime, one recovery tube per transfection reaction was prepared by mixing 125 µl fresh RBCs with 500 µl cRPMI. Additionally, the transfection mixtures per reaction were prepared by mixing 100 µl of the respective nucleofection solution (depending on nucleofactor used) with the respective plasmid solved in TE buffer (see chapter 4.2.1.5) in a separate tube each. At the end of the incubation, parasites were divided into separate tubes according to the number of transfection reactions, pelleted at 400g for 2 min and the supernatant was carefully removed as much as possible. Then 100 µl of the transfection reaction was transferred to the parasites, carefully mixed by aspirating slowly, transferred to a transfection cuvette and immediately transfected using program U-033 for the Lonza 2b and program FP-158 for the Lonza 4D nucleofactor. After nucleofection, the parasites were immediately aspirated with a provided plastic Pasteur pipette and transferred to the prewarmed recovery tube. For the recovery period of 30-45 min, tubes were sealed with Parafilm (to prevent them from opening and mitigate the risk of contamination) and placed in the ThermoMixer C at 37°C and shaking at 650 rpm. After recovery, the whole content of the tubes was transferred to a well of a 6-well plate containing 4.3 ml cRPMI and a thin blood smear was made from the culture as described above to check parasite survival. After 24 h, WR99210 was added to an end concentration of 2 or 2.5 nM. Following transfection, the growth medium was exchanged daily for up to 7 days unless the culture needed splitting before.

4.2.1.7 Parasite growth assays

Parasitemia of cultures that were included in the respective growth assay for knockdown validation was determined by counting iRBCs on thin blood smears imaged with the tabletop Keyence BZ-X800 microscope by eye and determining the number of RBCs in the same fields of view using the respective software. Afterwards, the parasitemia in all cultures were adjusted to be equal (0.3-0.4%, depending on the assay) and parasite cultures were transferred to a 6-well plate and placed in the incubator (see chapter 4.2.2.1). Thin blood smears were prepared in the beginning of each IDC for 2 cycles and the parasitemia determined the same way as before. Parasitemia values of each replicate were plotted in GraphPad Prism, log-transformed to fit a simple linear regression to these values. The slopes of these linear regressions was determined and log-transformed back which yielded the growth rate values for each replicate. These growth rate values were analyzed for significance with an ordinary one-way ANOVA using Dunnett's multiple comparisons test.

For measuring parasitemia to determine the IC₅₀ of WR99210 for dCas9 parasite lines sensitive or resistant to the drug, the respective parasite lines were grown in a 96-well plate for 3 cycles and treated with varying concentrations of WR99210 (0.00, 0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00 nM). Subsequently, they were prepared for flow cytometry analysis as described below (chapter 4.2.2) with the exception of staining parasite nuclei with 1:2000 SYBR Green I in PBS instead of DRAQ5. Flow cytometry analysis was performed as described below. The parasitemia values were plotted in GraphPad Prism to determine the drug's IC₅₀.

4.2.2 Flow cytometry

To prepare *P. knowlesi* samples for flow cytometry, 50 µl of normal 2.5% hematocrit cultures were transferred to a U-bottom 96-well plate with wells already containing 150 µl Dulbecco's phosphate buffered saline (DPBS). Parasitized RBCs were pelleted at 1500 rpm for 2 min and the supernatant removed. For fixing the cells, 4% paraformaldehyde (PFA) solution was freshly diluted from a 16% PFA stock with DPBS and glutaraldehyde added to 0.0075%. Accordingly, cells were resuspended in 200 µl of this fixing solution and incubated for 45 min at room temperature or overnight at 4°C. Afterwards, samples were pelleted again at 1500 rpm for 2 min, washed 1x with 200 µl DPBS and resuspended again in 200 µl DPBS for storage at 4°C until further use.

Before flow cytometry measurements, an aliquot of each well (e.g. 50 or 100 μ l) was transferred to a new U-bottom 96-well plate, pelleted at 1500 rpm for 2 min and the supernatant removed. Cells were resuspended in 200 μ l 0.1% Triton X-100, which was freshly diluted from a 10% stock with DPBS, and incubated for 8 min at room temperature for permeabilization. Permeabilized cells were pelleted and washed 1x with 200 μ l DPBS as before. For determining GFP(+)-cells, parasite cells had to be stained with a nuclear dye that is not green-fluorescent. Therefore, I used DRAQ5, which can be measured with APC-Cy7. The DRAQ5 dye was freshly diluted 1:1000 in DPBS and cells were resuspended in 100 μ l of this staining solution for at least 10 min at 37°C in the dark. Stained cells were washed 1x with DPBS as before and resuspended in 200 μ l DPBS. The samples were measured with either a FACSCanto II or a FACSCelesta. After gating for the bulk of cells, then for single cells, gates were set to capture nucleated cells by using iRBCs that had not been stained with DRAQ5. Similarly, gates were set to capture GFP(+) cells by using iRBCs that were GFP(-). When both GFP(+) and mScarlet(+) cells were measured, compensation of the voltage was set so that there was no bleed-through between the FITC (green) and PE (red) channels. Data obtained by flow cytometry was analyzed using the FlowJo software.

4.2.3 Immunofluorescence assay

For immunofluorescence staining, the general procedure described in (Mehnert, Simon and Guizetti, 2019) was used. Infected RBCs were seeded onto 8-well ibidi-treat slides as a monolayer. For this, 100 μ l of culture had been pelleted at 800g for 1 min and washed 3x with iRPMI to remove lipids and proteins from cRPMI. Cells were then allowed to settle to the bottom for 10 min at 37°C and unbound cells were subsequently removed by gently aspirating the cell solution up and down with a micropipette. After washing the cells quickly with phosphate buffered saline (PBS), they were fixed with freshly prepared 4% PFA in PBS for 20 min at 37°C. Cells were washed 2x with PBS and either stored at 4°C or immediately stained for immunofluorescence analysis (IFA). First, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and washed 3x with PBS. Then, free aldehyde groups were quenched by incubating the cells with ~0.1 mg/ml NaBH₄ in PBS for 10 min and washing 3x with PBS. To block unspecific binding sites of the antibodies, cells were incubated in 3% (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature. Primary antibodies were diluted in BSA/PBS as described previously (chapter 4.1.5), added to the samples and incubated for at least 1 h. To wash away unbound antibodies, cells were washed 3x with 0.5% Tween-20 in PBS (PBS-T) before adding secondary antibodies and Hoechst dye diluted in BSA/PBS as described

previously (chapter 4.1.5). Samples were incubated with secondary antibodies for at least 30 min in the dark. Cells were again washed 2x with 0.5% PBS-T and 1x with PBS and stored at 4°C until use. IFA images were acquired with the SP8 point laser scanning confocal microscope.

4.2.4 Molecular cloning

4.2.4.1 Restriction digestion

Plasmid DNA (2 µg) was digested in a 50 µl volume reaction, using 5 µl 10x CutSmart buffer and 1 µl of the respective restriction enzymes each. The digest was incubated at 37°C for 1-3 h, depending on the enzymes.

4.2.4.2 Oligonucleotide annealing

For sgRNA construct assembly, oligonucleotide (oligo) sequences were ordered in desalted and lyophilized form and resuspended in nuclease-free water to a stock concentration of 100 µM. Oligos with homologous sequences were annealed at 20 µM diluted in TE buffer (pH=8) in a 50 µl reaction using a thermocycler. During the annealing reaction, DNA was denatured at 95°C for 5 min and then the temperature was decreased to 25°C in a ramp-down of 0.1°C/s. The annealed oligos were diluted 1:40 in cold TE buffer and used in Gibson assembly (see Gibson assembly) to assemble sgRNA constructs.

4.2.4.3 Polymerase chain reaction

Polymerase chain reactions (PCRs) were generally performed using Phusion High-Fidelity (HF) DNA polymerase according to manufacturer's guidelines:

Tab. 4.16: PCR reaction.

Component	Final concentration
5x Phusion HF buffer	1x
10 mM dNTPs	200 µM
10 µM forward primer	0.5 µM
10 µM reverse primer	0.5 µM
Phusion DNA polymerase	1 unit/50 µl PCR reaction

Generally, reactions were performed in 50 µl and ca. 5 ng of plasmid DNA was used as template. Nuclease-free water was used to fill up the reactions to 50 µl. Primers were ordered in desalted and lyophilized form and resuspended in nuclease-free water to a stock concentration of 100 µM. All reactions were assembled on ice and the DNA polymerase was added last.

Thermocycler reactions were used according to manufacturer's guidelines:

Tab. 4.17: Thermocycler PCR cycles.

Step	Temperature	Time
Initial denaturation	98°C	30 s
Denaturation	95°C	5-10 s
Annealing	50-55°C	10-30 s
Elongation	72°C	15-120 s (30-60 s per kb)
Final extension	72°C	10 min
Cooling	4°C	Indefinitely

The annealing temperature depended on the primers used and was calculated using the NEB T_m calculator (<https://tmcaculator.neb.com/#!/main>). The standard elongation/extension temperature of 72°C was decreased down to 62°C for untranslated regions (UTRs) due to the higher AT-content.

4.2.4.4 Agarose gel electrophoresis and DNA purification from agarose gels

Agarose gel electrophoresis was performed to separate DNA fragments according to size, either to visualize them or to purify them afterwards. Depending on the size of the fragments, DNA was either separated in 0.8% (w/v) or 2% (w/v) agarose gels. DNA samples were mixed with 6x purple loading dye that had been supplemented with 1:500 Midori Green DNA dye. Again, depending on the size of the fragments to be analyzed, either 1 kb or 100 bp DNA ladder was loaded alongside the samples and run at 135 V, 130 W and 1000 mA for ca. 25 min. Separated DNA bands were visualized using a UV table and imaged with a camera.

The bands of the desired size were cut out with a scalpel and subsequently purified using the High Pure PCR Product Purification kit according to manufacturer's guidelines. DNA was eluted in 20 µl H₂O.

4.2.4.5 Gibson assembly and bacterial transformation

Plasmids were assembled via Gibson assembly using 2-3 fragments. If possible, 100 ng of linearized backbone (or the maximum amount for a 20 µl reaction) was mixed with the insert in a 1:2 molar ratio and either 5 or 10 µl of 2x Gibson assembly master mix (Hifi Assembly Master Mix) were added to make either a 10 or 20 µl reaction. The reaction was incubated at 50°C for 15 min.

The assembled plasmids were then transformed into chemically competent DH5α *E. coli* cells. For this, 30 µl of cells were mixed with 2 µl of the Gibson assembly reaction mix and incubated

for 30 min on ice. The cells were then heat-shocked at 42°C for 30 s and incubated again on ice for 2 min. Subsequently, the cells were mixed with 350 µl SOC outgrowth medium and incubated at 37°C and 130 rpm for 1 h. Finally, the cells were spread on prewarmed LB-Amp plates and carefully air-dried before the plates were incubated at 37°C overnight.

4.2.4.6 Miniprep and glycerol stocks

To be able to send assembled plasmids for sequencing to ensure correct plasmid sequences, 3 ml bacterial cultures were prepared by picking a single colony from a plate and incubating the culture at 37°C overnight at 130 rpm. The plasmids were then extracted from 2 ml bacterial culture using the GenElute HP Plasmid Miniprep Kit according to manufacturer's guidelines. The concentration of the eluted plasmid was measured using a NanoPhotometer NP80.

From the remaining bacterial culture 500 µl were mixed with 500 µl of a 50% glycerol solution (diluted in ddH₂O) and transferred into a cryotube that was subsequently placed at -80°C for long-term storage.

4.2.4.7 Sequencing

Plasmid sequences were sent to Eurofins Scientific via their SupremeRun Tube overnight service. The plasmids were prepared according to their instructions (20 µl at 50-100 ng/µl) and 20 µl sequencing primers were added at 10 µM each. The sequencing results were analyzed with ApE and SnapGene Viewer.

4.2.5 DNA extraction

Parasite (genomic) DNA was extracted using the DNeasy Blood and Tissue Kit according to manufacturer's guidelines for using non-nucleated blood. Generally, 200 µl pelleted iRBCs were either used directly or stored at -20°C until use. For the samples taken during the screen, only 20 µl packed iRBCs were used and the reagent volumes adjusted accordingly. DNA was eluted in 30-50 µl.

4.2.6 RNA extraction and cDNA synthesis

Since the transcripts that were to be analyzed were expressed primarily in the schizont-stage, parasites were synchronized to that stage with 2x Nycodenz gradient centrifugations in consecutive cycles. After the second Nycodenz centrifugation, instead of resuspending the

schizont-layer in RBCs and medium for standard culture, the washed and pelleted schizonts were immediately frozen at -20°C in a microfuge tube or used directly for RNA extraction. If samples had been frozen, they were thawed on ice, followed by RNA extraction using the RNeasy Mini kit and according to manufacturer's guidelines. For this, the required volume of RLT lysis buffer (depended on the volume of sample) was freshly supplemented with 1:50 β -mercaptoethanol and added to the cells. Then, 1 volume of 70% ethanol diluted with RNase-free water was added and the lysate solution was added to a RNeasy spin column placed in a 2 ml tube followed by centrifugation at $\geq 8000g$ for 15 s. The column was washed as described in the manual and RNA was eluted in 30-50 μ l RNase-free water. The concentration of the RNA sample was measured with a NanoPhotometer NP80.

To remove any DNA that might have remained in the sample, DNase treatment using the TURBO DNA-free kit was used according to manufacturer's guidelines. Depending on the RNA yield, 2-5 μ g RNA were used in a 50 μ l reaction, which was filled up with RNase-free water if necessary. The sample was incubated with TURBO DNase for 20-30 min at 37°C and subsequently the DNase inactivation reagent was added (0.1 volume, at least 2 μ l) to stop the reaction. After 5 min incubation at room temperature, the inactivated DNase was pelleted at 10 000g for 90 s and the RNA in the supernatant was transferred to a fresh microfuge tube. The concentration of the RNA sample was again measured using the NanoPhotometer NP80 and the A260/A280 and A260/A230 purity ratios were noted. An A260/A280 ratio of <2 generally indicates possible contamination with proteins or organic compounds. Similarly, an A260/A230 ratio of 2.0-2.2 would be expected for "pure" RNA and a considerably lower ratio is indicative of contamination with TRIS, EDTA or buffer salts. Although RNA samples could be stored at -80°C, they were placed on ice and used immediately for cDNA synthesis.

For cDNA synthesis, the First Strand cDNA Synthesis Kit was used according to manufacturer's guidelines. If possible, 500 ng DNase-treated RNA (range of 250-500 ng is recommended) was used and "no template"- and "no reverse transcriptase"-controls were included. RNase-free PCR tubes were used and the reactions were assembled on ice. For non-specific cDNA synthesis, random hexamer primers were used. DNase-treated RNA, primers, 5x reaction buffer, RiboLock RNase inhibitor, 10 mM dNTP mix and MuLV reverse transcriptase were mixed in 20 μ l reactions. The reactions were incubated at 25°C for 5 min and at 37°C for 60 min before heating to 70°C for 5 min to stop the reaction using a thermocycler. The 20 μ l reactions were mixed with 1 ml of nuclease-free water to make a working stock for RT-qPCR. The cDNA samples were stored at -20°C for less than a week or immediately at -80°C.

4.2.7 Real-Time quantitative PCR

Real-Time quantitative PCR (RT-qPCR) reactions were always assembled on ice.

For measuring transcript levels of target genes, 20 µl reactions were assembled with 5 µl of cDNA working stock (see RNA extraction and cDNA synthesis; 250-500 ng DNase-treated RNA was used for cDNA synthesis in 20 µl reaction to which 1 ml nuclease-free water was added). The reactions therefore included: 10 µl 2x SsoAdvanced Universal SYBR Green Supermix, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 3 µl nuclease-free water and 5 µl cDNA working stock. I assembled a master mix containing the SYBR Green Supermix, the respective primers and nuclease-free water, which was first pipetted in the corresponding wells of a MicroAmp optical 96-well reaction plate. The cDNA was added in a second step before the plate was transferred into a QuantStudio 1 Real-Time PCR system. Transcript levels of the serine-tRNA-ligase gene (PKNH_0312700, PKA1H_030017700) were measured as the “housekeeping gene” control. Primer pairs are listed in Tab. 4.13 Transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

For RT-qPCR primers used for the readout of the screen, primer efficiencies were determined by measuring a plasmid dilution series (2, 20, 200, 2000 fg/well) for each primer. The threshold cycle (Ct) values were plotted, and the slope y of the resulting line was used to calculate the primer efficiency:

$$\text{Primer efficiency} = (10^{\frac{1}{y}} - 1) \times 100$$

The efficiencies were between 95-113%. The RT-qPCR reactions for determining primer efficiencies and the relative abundance ratios of the plasmids in the screen were assembled in 10 µl and the volume of the reagents adjusted accordingly. The relative abundance ratio of plasmids was determined from DNA extraction samples of the respective parasite culture and 2.5 µl of DNA elution was used. For detecting the respective sgRNA sequence of the constructs, each construct had a unique forward primer and the reverse primer bound in the plasmid backbone so that the same one could be used (primer 37). For normalization, the abundance of all plasmids was measured using primers amplifying the hDHFR cassette. The relative plasmid abundance was calculated as:

$$\Delta C_t = C_t(\text{specific}) - C_t(hDHFR)$$

$$\text{Relative abundance ratio} = 2^{-\Delta C_t}$$

The RT-qPCR cycling program consisted of 3 stages. In stage 1, the samples were heated to 50°C for 2 min and then denatured at 95°C for 10 min. In stage 2, 35 cycles of denaturation at

95°C for 15 s, followed by annealing at 60°C for 60 s were repeated. In stage 3, the melt curve of the amplified samples was determined by first denaturing at 95°C for 15 s again and then cooling to 60°C in increment steps.

4.2.8 Small-scale knockdown screen

Synchronizing parasites for transfection and the transfection itself were performed as described before. The plasmid input consisted of either 20 µg of all 20 sgRNA constructs (i.e., 1 µg of each construct; “Lo” condition) or 200 µg of all 20 sgRNA constructs (i.e., 10 µg of each construct; “Lo” condition), each diluted in 10 µl TE buffer. In the first replicate, 1.7×10^8 schizont-stage parasites were used per reaction and in the second replicate 8.4×10^8 parasites per reaction.

After transfection, samples of 800 µl culture were taken (corresponding to 20 µl packed iRBCs) at the pre-set time points of cycle 1 (directly after recovery from transfection), cycle 3, cycle 7 and cycle 10. Since there was a growth lag phase, it was decided to take at least one more sample after parasites entered exponential growth. In the first replicate, the samples were washed 3x with iRPMI and the iRBC pellets were immediately frozen at -20°C. Due to excess plasmid background in the samples, the sampling procedure was extended for the second replicate. Therefore, after washing, the pellet was resuspended in 909 µl iRPMI and 91 µl 10x DNase I buffer and 7.5 µl 2U/µl DNase I (15 U/ml final concentration) were added. The sample was then split, and half (500 µl) was just incubated at room temperature for 2 min before stopping the DNase digestion by adding 5 µl 0.5 M EDTA. This sample was then pelleted at 800g for 2 min, the supernatant removed, and the pellet incubated at 75°C for 5 min. The other half of the sample (500 µl) was mixed with 500 µl 0.05% Saponin for max. 5 min before EDTA was added as before. This sample was pelleted at 3000g for 6 min and incubated at 75°C for 5 min. Samples were immediately frozen at -20°C and stored until DNA extraction. In the first 3 cycles, cultures were still relatively synchronized so that samples were taken when most parasites were in the ring-stage to minimize confounding effects. It should be noted that between cycle 7 and 10 a 1:2 split of the culture was performed to provide fresh RBCs.

Parasite DNA was extracted from the samples as described before and the relative plasmid abundance was measured by RT-qPCR as described before.

The abundance of each specific plasmid was first normalized to the abundance of all plasmids using a primer pair that bound in the hDHFR resistance cassette (primer pair 21 and 22,

Tab. 4.13 in chapter 4.1.8) present in all sgRNA constructs. From this, the relative abundance ratio of each sgRNA construct was calculated as follows:

$$\Delta Ct = Ct(specific) - Ct(hDHFR)$$

$$Relative\ abundance\ ratio = 2^{-\Delta Ct}$$

5. References

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I would like to say a special thank you to Marius for being the absolute best student anyone could ask for. It was truly a joy and a privilege to supervise you in the lab and I am glad we have become friends this way. Thank you for your openness and honesty and, of course, for pulling some night shifts with me (on occasion with Vanessa and Yannik, aka Transfection shenanigans gang).

Another special thank you to the members of the Parasitology representatives. I truly enjoyed working together with you on exciting and important projects.

I am also grateful to everyone who has showed up, be it once or every time, to our Parasitology peer mentoring group. I feel fortunate to have learned and shared wisdom alongside you. Thank you to Guille and Violeta for helping me start this. I am also grateful to Guille for becoming a good friend and for your valuable feedback on this thesis.

I would also like to extend my gratitude to Prof. Freddy Frischknecht and the members of his lab for their help and support. A special thanks to Franzi for help and guidance with data analysis. I am also grateful to Annika and Kevin for the nice time we had at the ICOPA conference in Copenhagen. Thank you to members of Prof. Fackler's group and to the MCTU lab for letting me use their flow cytometry equipment and for helping me whenever it was not working properly. Thank you also to Nathalia and Carrie for your help and support and great conversations. A special thanks to Miriam and Sandra for being the best secretaries and always showing so much support!

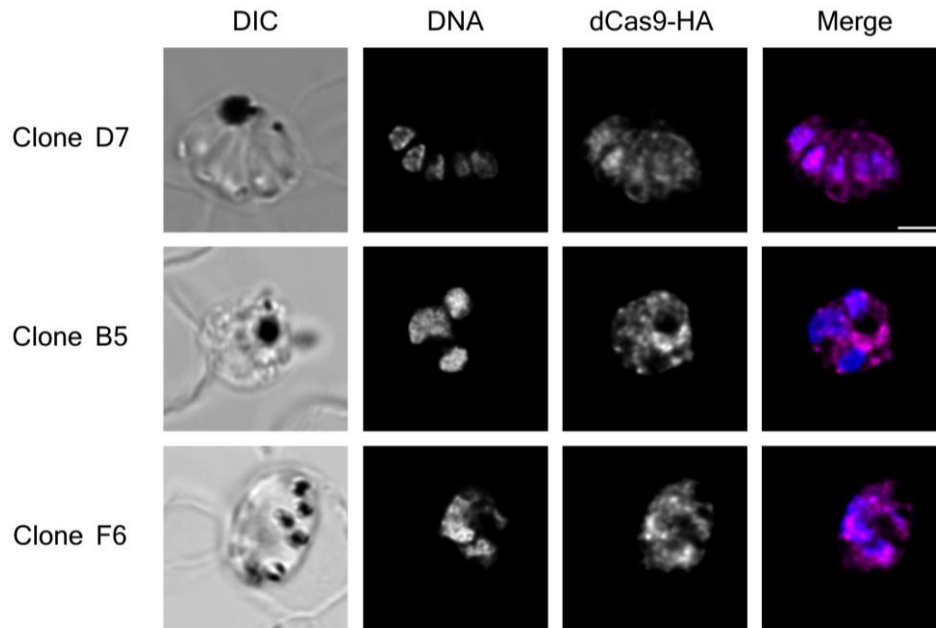
A big thank you to my family, to Elke, Sonja, Stephan, Alex, Emilia and Nakoa. Thank you for always believing that I could achieve what I set my mind to.

Thank you to Tatjana, Melli and Michaela for your great friendship, for our brunch dates and for inspiring me to see the best in life.

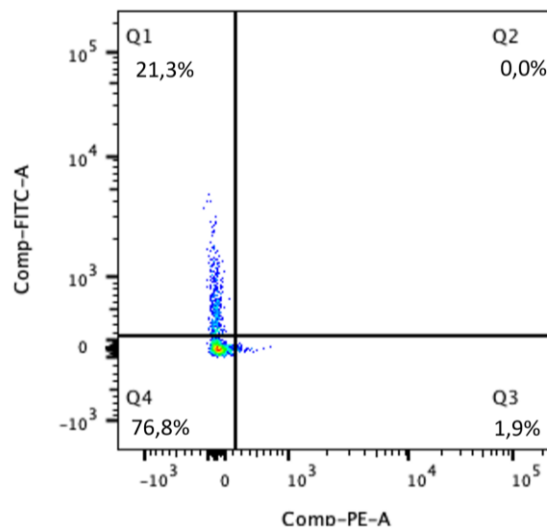
And finally, I would like to express my immense gratitude to Markus for, well, for everything. Thank you for always having my back, for making me laugh, for your patience and your understanding during this extraordinary phase of my life.

There are so many more people I have to thank for contributing to and enriching my PhD experience over the last four and a half years and I am sorry for not being able to name everyone here. And although she has not directly been a part of this PhD experience, I would like to thank Betty Henderson because without her I probably would have never found my way to exploring the fascinating world of biology. Thank you for being the best teacher, for encouraging me and for lighting a spark of inspiration that, 15 years later, led me here.

7. Supplementary information



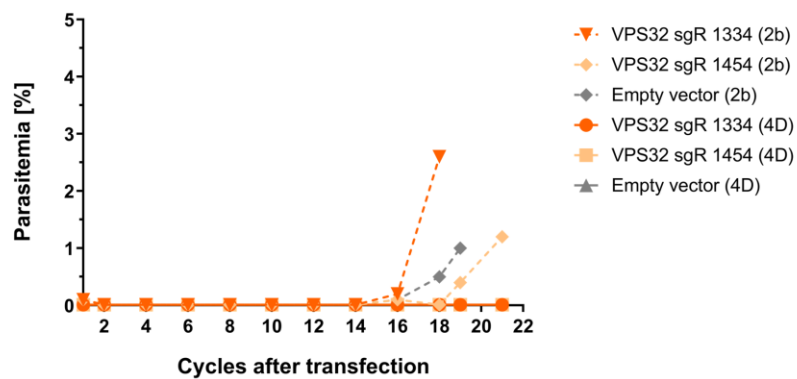
Suppl. Fig. 1: Immunofluorescence analysis of dCas9-3xHA expression and localization in dCas9 clones. DIC: Differential interference contrast. Blue channel: Hoechst, red channel: anti-HA. Scale bar 2 μ m.



Suppl. Fig. 2: dCas9 parasites transfected with reporter constructs expressing either GFP or mScarlet only showed expression of one of the reporters. Parasites of the dCas9 line were transfected with a plasmid mix containing both pPkconGFP and pPkconmScarlet plasmids. Thirteen cycles after transfection, a sample was taken and the GFP(+) (FITC) and mScarlet(+) (PE) fractions of the parasite population were measured using flow cytometry.

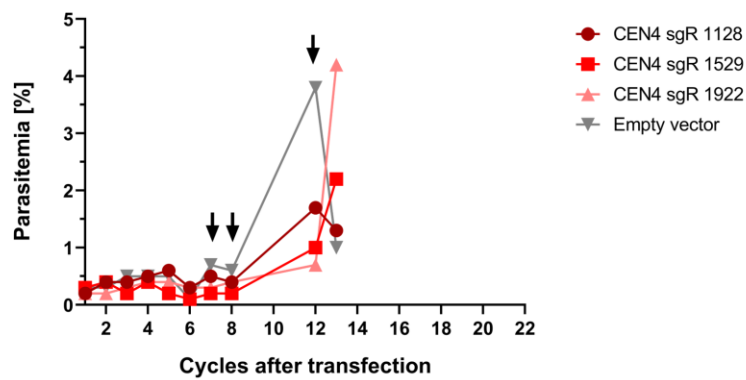
A

Transfection VPS32 sgRNA constructs (TF 5)



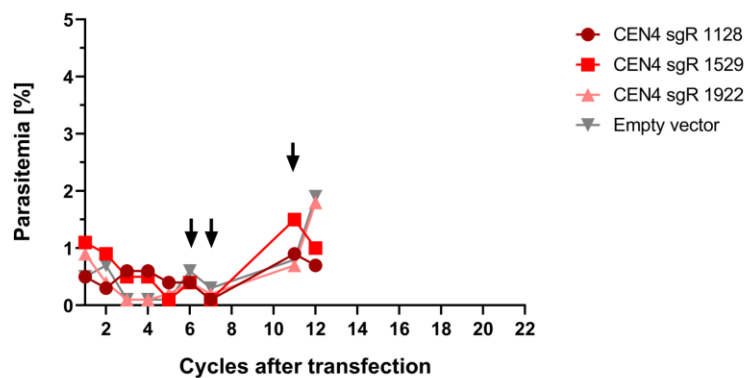
B

Transfection CEN4 sgRNA constructs (n1)



C

Transfection CEN4 sgRNA constructs (n2)



Suppl. Fig. 3: Growth of parasite lines transfected with sgRNA constructs exhibited a lag phase of variable length. Parasitemia of cultures transfected with sgRNAs targeting VPS32 (A) or CEN4 (B) and (C), was

determined by counting parasites on thin blood smears using a tabletop microscope. Black arrows indicate culture splits.

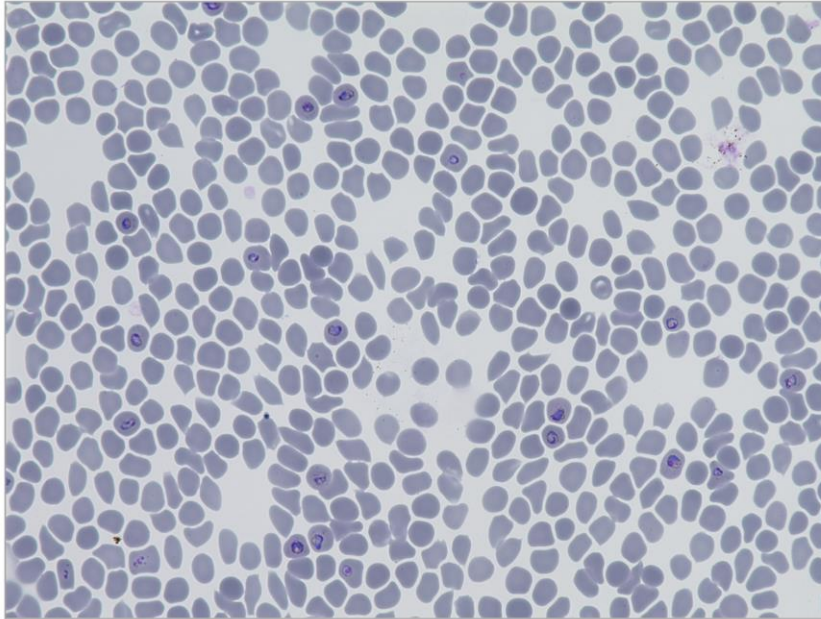
Suppl. Tab. 1: Sequences (either 400 bp or 100 bp) flanking TSSs identified by StringTie were extracted and recurring motifs were detected with DREME and MEME.

400 bp sequences			
	Motif	Sequences positive for motif	Statistical significance (e-value)
MEME	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTYHNY	1176/1804	8,4e-1116
	GRAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	865/1804	5,3e-325
	TTTTYYTTTTYYYYTYYYYCHYYTTKY	1288/ 1804	6,00E-118
DREME	ATATRYAT	708/1804	2,40E-133
	RGGGGRR	984/804	6,30E-100
	RTAYAY	1493/1804	9,50E-90
100 bp sequences			
	Motif	Sequences positive for motif	Statistical significance (e-value)
MEME	TTTTTTTTTTTTTTTTTTTTTTTTTTTTT	677/1804	4,6e-2066
	RAAAWRRARRAAAAA	638/1804	6,70E-201
	YCCCCYCCCCSHNBYKBHSC	182/1804	1,60E-74
DREME	GRRGRA	930/1804	3,30E-40
	TRTRYA	749/1804	1,10E-32
	CTHCAY	405/1804	5,00E-17

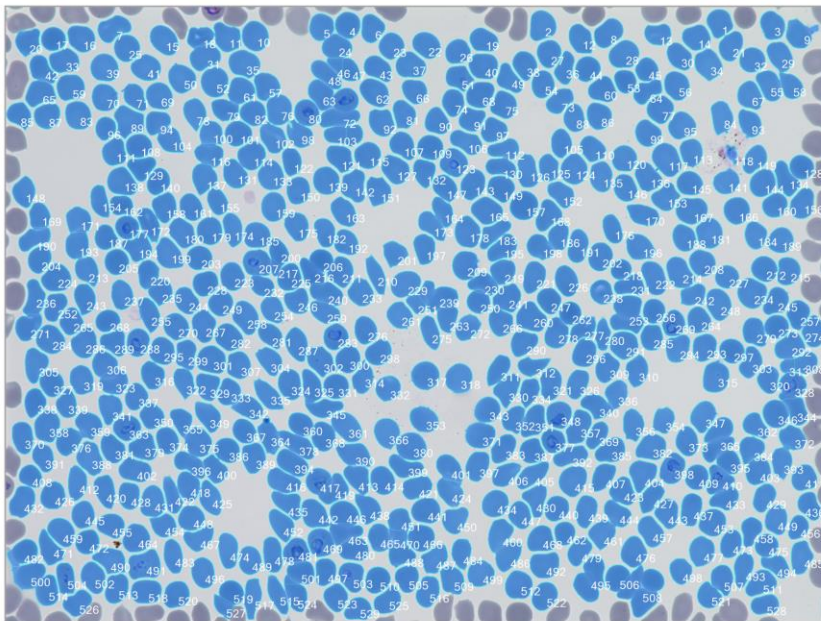
Suppl. Tab. 2: Criteria according to which sgRNA list was assembled.

Parameters	Filtration criteria
Total score	>0.1
Efficiency score (Doench et al., 2014)	"keep all"
Efficiency score (CRISPRater)	>0.59
Number of on-targets (perfect match)	"keep all"
Number of on-targets (imperfect match)	"keep all"
Number of off-targets (perfect match)	Equal 0
Number of off-targets (imperfect match)	Equal 0
GC-content	>30%

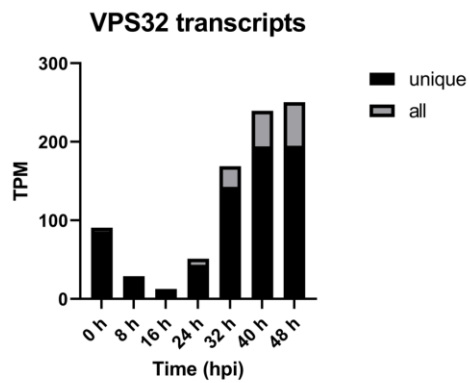
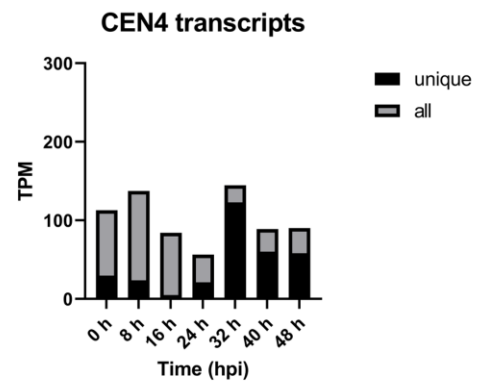
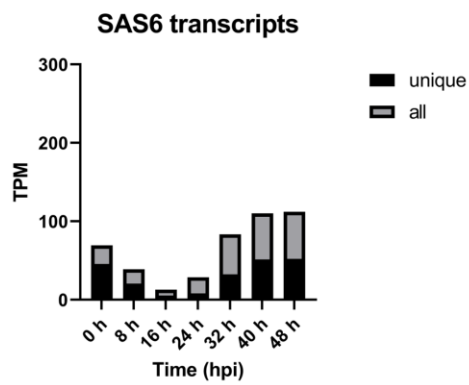
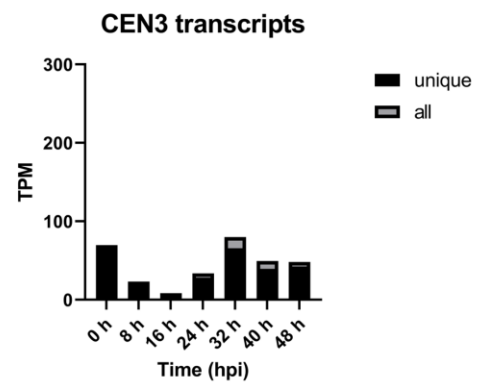
A



B

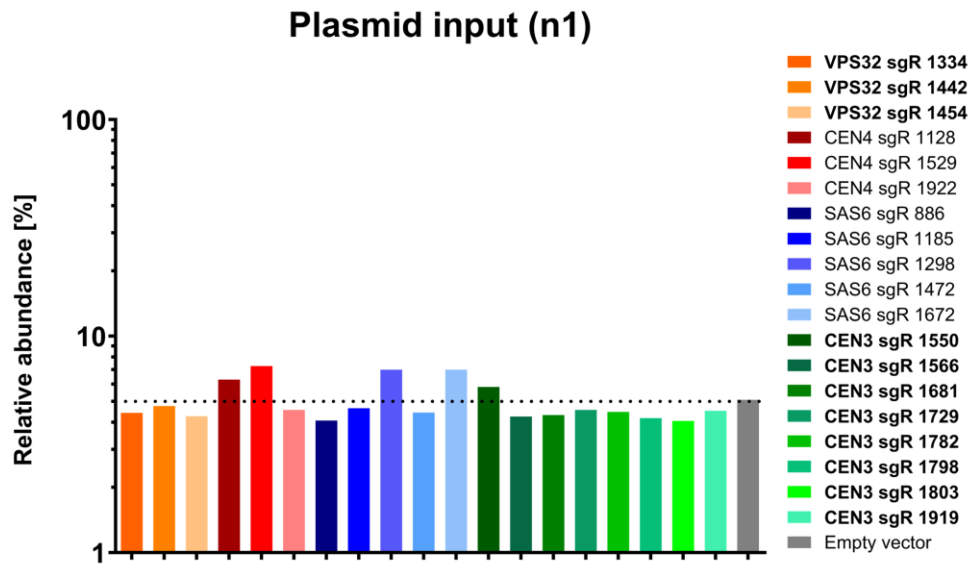


Suppl. Fig. 4: Determining parasitemia with images of thin blood smears. Thin blood smears were dried, fixed with methanol and stained with Hemacolor solution I and II. The sample slide was then imaged using a tabletop Keyence microscope (A) and parasites were counted by eye. To determine the number of RBCs in the same field of view/image, a mask was generated with the accompanying software (B) that could be adjusted for each slide.

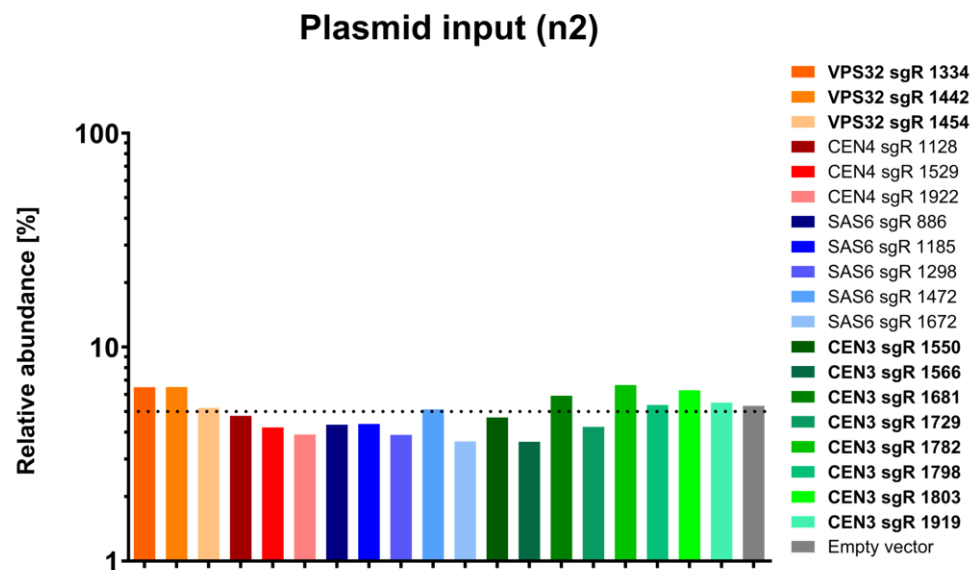
A**B****C****D**

Suppl. Fig. 5: Transcription profiles of VPS32, CEN4, SAS6 and CEN3 genes in *P. falciparum*. The transcription profiles of the VPS32 (**A**), CEN4 (**B**), SAS6 (**C**) and CEN3 (**D**) are shown as determined for the *P. falciparum* strain in (Otto *et al.*, 2010). In this study, samples were taken every 8 h during asexual blood-stage growth and the number of transcripts measured via RNA-sequencing. TPM: transcripts per million.

A

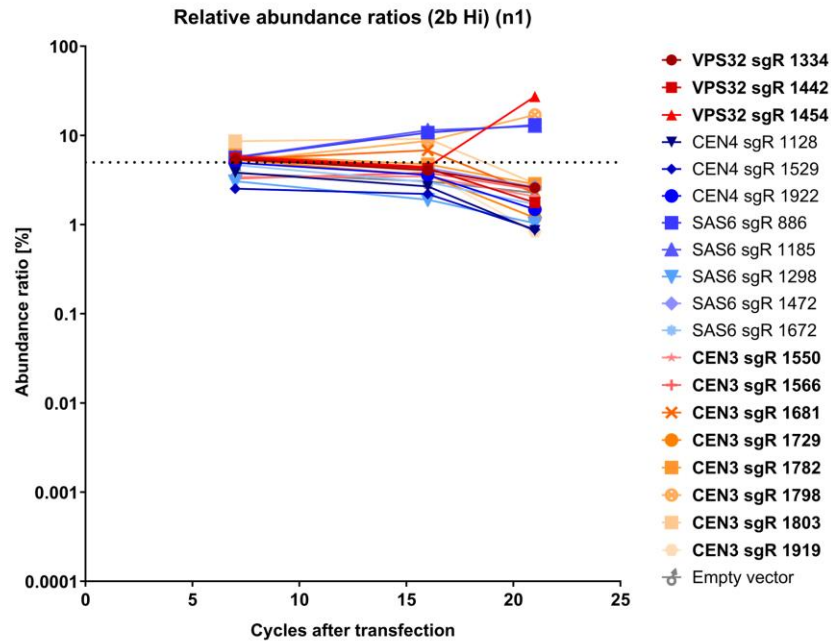


B

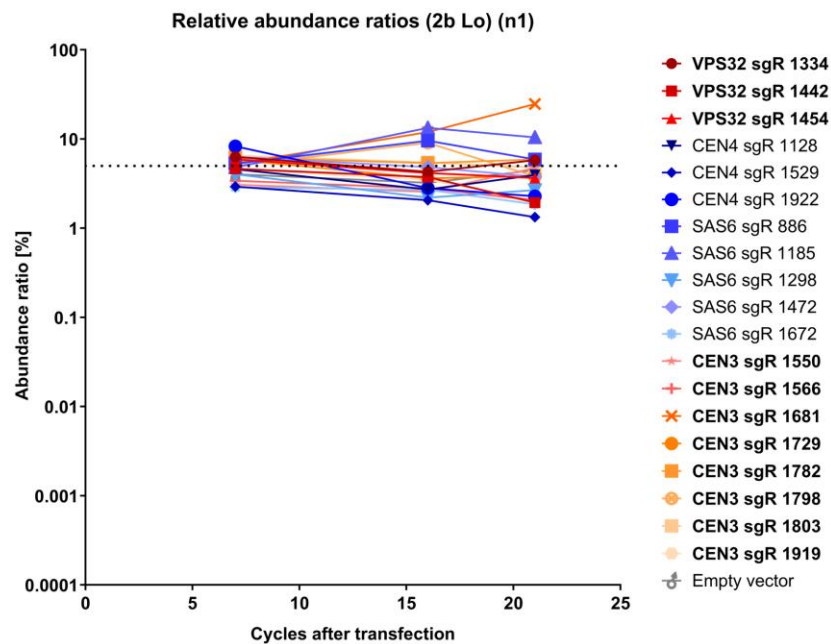


Suppl. Fig. 6: Input sample used for transfecting proof-of-principle screen showed uniform distribution of plasmids. A sample was taken from the input that was transfected into parasites during the first (A) and second (B) replicate of the screen and their relative abundance ratio determined using RT-qPCR. The dotted line at 5% relative abundance represents a state where all 20 constructs are equally abundant. sgRNA IDs for sgRNAs targeting essential genes are shown in bold in the legends.

A

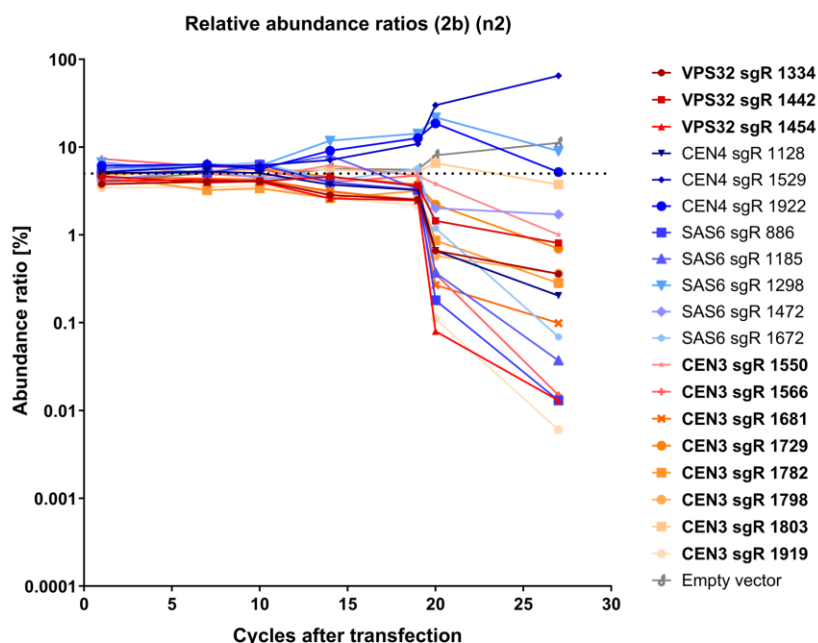


B

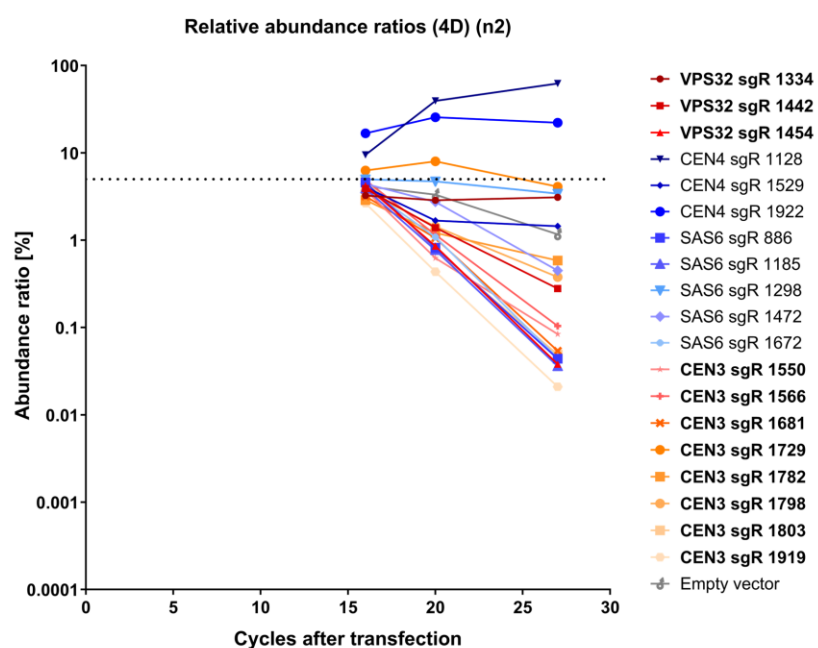


Suppl. Fig. 7: The relative abundance ratios of constructs in the “2b Hi” and “2b Lo” conditions of the first replicate did not show variation in the first few time point samples. A plasmid library comprising 20 constructs was transfected into segmented schizont-stage parasites. From the parasite samples taken at several time points, DNA was extracted and used for RT-qPCR to determine the constructs’ abundance. The Ct values were used to calculate relative abundance ratios for each construct which represents its relative abundance in the plasmid pool as a percentage. Since there were 20 constructs in the pool, if all the constructs were present at uniform abundance, the relative abundance ratios approximated 5% (dotted line). sgRNA IDs for sgRNAs targeting essential genes are shown in bold in the legends.

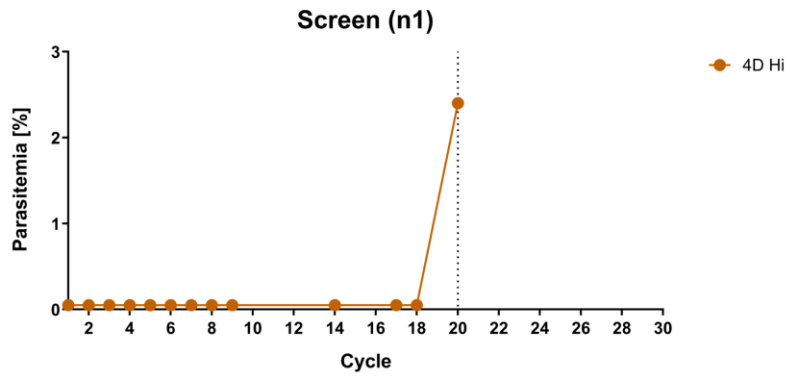
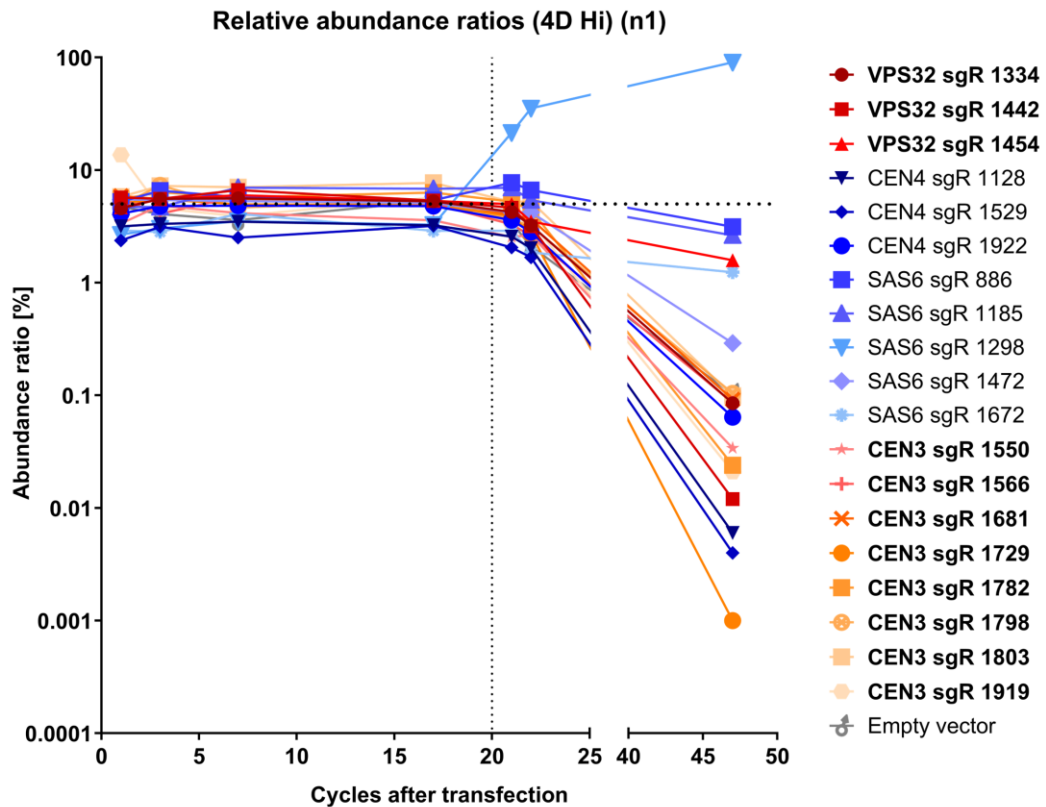
A



B

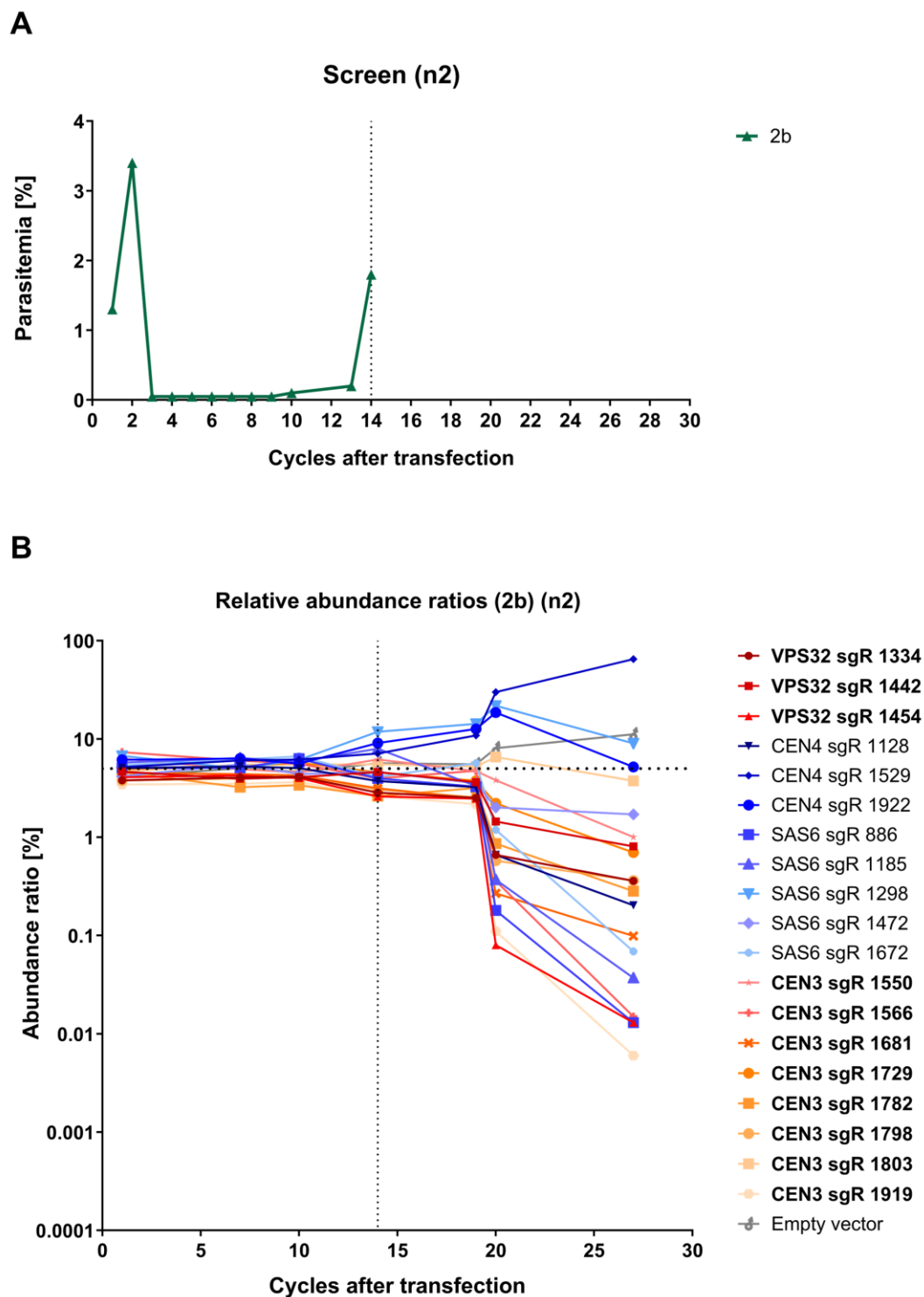


Suppl. Fig. 8: The relative abundance ratios of constructs in the “2b” and “4D” conditions of the second replicate did not show variation in the first few time point samples. Plasmid library comprising 20 constructs was transfected into segmented schizont-stage parasites using the 2b (**A**) or 4D (**B**) nucleofectors. From the parasite samples taken at several time points, DNA was extracted and used for RT-qPCR to determine the constructs’ abundance. The Ct values were used to calculate relative abundance ratios for each construct which represents its relative abundance in the plasmid pool as a percentage. Since there were 20 constructs in the pool, if all the constructs were present at uniform abundance, the relative abundance ratios approximated 5% (dotted line). sgRNA IDs for sgRNAs targeting essential genes are shown in bold in the legends.

A**B**

Suppl. Fig. 9: The time point when the relative abundance ratios of construct started to vary in the “4D Hi” condition correlated well with the time point when the respective parasite culture entered exponential growth. (A) Growth of the 4D Hi parasite line in the first replicate of the screen. Parasitemia was determined by counting parasites on thin blood smears. (B) Plasmid library comprising 20 constructs was transfected into segmented schizont-stage parasites using the 4D nucleofector. From the parasite samples taken at several time points, DNA was extracted and used for RT-qPCR to determine the constructs’ abundance. The Ct values were used to calculate relative abundance ratios for each construct which represents its relative abundance in the plasmid pool as a percentage. Since there were 20 constructs in the pool, if all the constructs were present at uniform abundance, the relative abundance ratios approximated 5% (horizontal dotted line). sgRNA IDs for sgRNAs

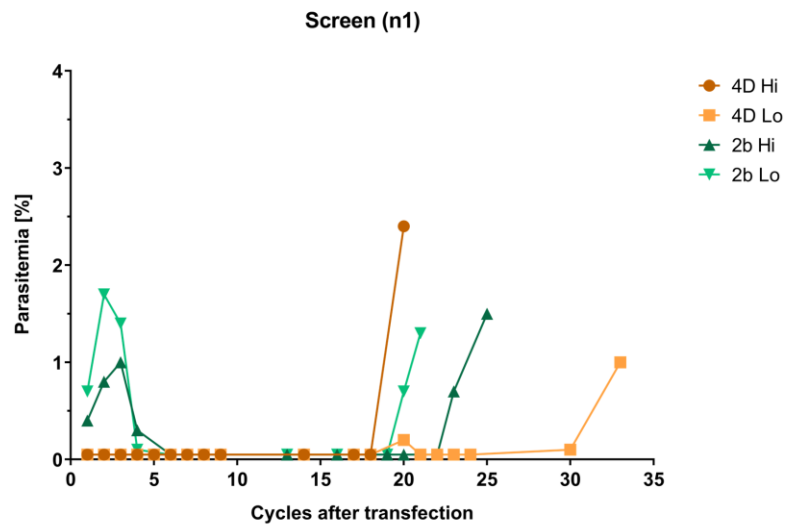
targeting essential genes are shown in bold in the legends. The vertical dotted lines indicate the time point when parasites entered exponential growth (A) or when relative abundance ratios started to vary (B).



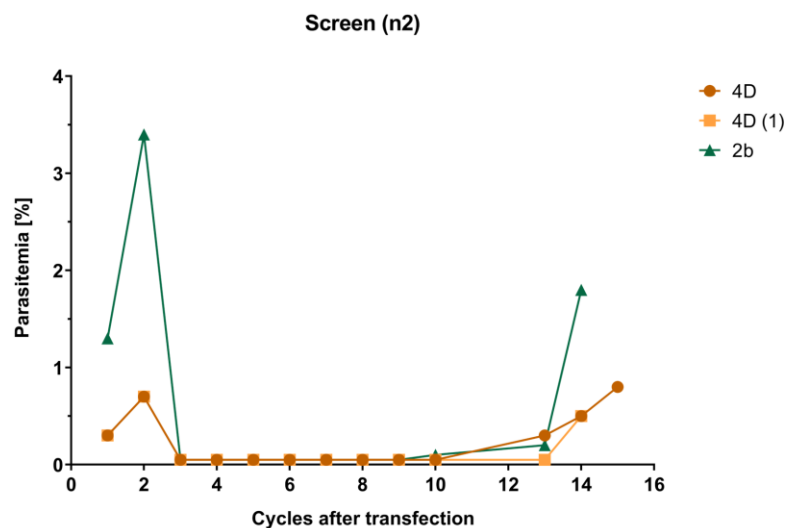
Suppl. Fig. 10: The time point when the relative abundance ratios of construct started to vary in the “2b” condition correlated well with the time point when the respective parasite culture entered exponential growth. (A) Growth of the 2b parasite line in the first replicate of the screen. Parasitemia was determined by

counting parasites on thin blood smears. **(B)** Plasmid library comprising 20 constructs was transfected into segmented schizont-stage parasites using the 2b nucleofector. From the parasite samples taken at several time points, DNA was extracted and used for RT-qPCR to determine the constructs' abundance. The Ct values were used to calculate relative abundance ratios for each construct which represents its relative abundance in the plasmid pool as a percentage. Since there were 20 constructs in the pool, if all the constructs were present at uniform abundance, the relative abundance ratios approximated 5% (horizontal dotted line). sgRNA IDs for sgRNAs targeting essential genes are shown in bold in the legends. The vertical dotted lines indicate the time point when parasites entered exponential growth **(A)** or when relative abundance ratios started to vary **(B)**.

A



B



Suppl. Fig. 11: Growth of parasite lines transfected with plasmid library exhibited a lag phase of variable length. Parasitemia of cultures transfected with plasmid library comprising 20 constructs targeting the VPS32, CEN4, SAS6 and CEN3 genes was determined by counting parasites on thin blood smears using a tabletop microscope. **(A)** First replicate of the screen. **(B)** Second replicate of the screen.