SHYMAA ALI SAEED ALI

METHOD FOR DELETION OF VARIANT SURFACE ANTIGEN GENES AT SUBTELOMERIC REGION OF *PLASMODIUM FALCIPARUM*

GRADUATE SCHOOL OF FRONTIER

BIOSCIENCES The UNIVERSITY of OSAKA

2025

METHOD FOR DELETION OF VARIANT SURFACE ANTIGEN GENES AT SUBTELOMERIC REGION OF *PLASMODIUM FALCIPARUM*

DOCTORAL DISSERTATION

Submitted as partial fulfillment of the requirement to obtain

Doctor of Philosophy Degree from Graduate School of Frontier

Biosciences, The University of Osaka, Japan

September 2025

Shymaa Ali Saeed Ali

32A20073

Prof. Tetsuya Iida , Ph.D.

Supervisor

ABSTRACT

METHOD FOR DELETION OF VARIANT SURFACE ANTIGEN GENES AT SUBTELOMERIC REGION OF *PLASMODIUM FALCIPARUM*

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Plasmodium falciparum expresses variant surface antigens (VSAs), including PfEMP1, RIFIN, and STEVOR, on the surface of infected red blood cells. These antigens interact with host receptors on vascular endothelial and immune cells, contributing to both parasite pathogenicity and immune evasion. VSAs are encoded by large multigene families, comprising dozens to hundreds of genes located primarily in heterochromatic regions such as subtelomeric domains, which are notoriously refractory to genetic manipulation. In addition, because *P. falciparum* parasites undergo antigenic variation by randomly switching VSA expression, it is challenging to use parasites that stably express target VSAs for experimental purposes. As a result, functional characterization of these VSAs has been limited, despite their well-established clinical significance. In this study, I present a novel method for targeted deletion of subtelomeric regions in *P. falciparum* chromosomes by combining the heterochromatin-accessible AsCas12a-UL nuclease with telomere healing. Using this approach, I successfully deleted both subtelomeric regions of chromosome 2. Furthermore, I achieved simultaneous removal of up to seven subtelomeric regions using tandemly arrayed crRNAs, with an efficiency exceeding 85%. This method provides a powerful tool for generating VSA-null parasites, facilitating precise genetic dissection of individual VSA gene families and their roles in host–parasite interactions.

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CHAPTER 1 - GENERAL INTRODUCTION

1.1 Malaria

Malaria remains one of the most significant parasitic diseases affecting humans due to its global prevalence and its strong association with high morbidity and mortality rates, particularly in sub-Saharan African region. The term malaria originates from the Italian words mal'aria, meaning "bad air," reflecting the historical belief that the disease transmission was associated with the dirty air of marshy regions. The causative agents of malaria are protozoan parasites belonging to the genus *Plasmodium*, which are transmitted to the host (human) through the bite of the vector which is infected female *Anopheles* mosquitoes(1),(2).

1.1.1 Epidemiology

Geographically, malaria is prevalent in Central and South America, Africa, the Middle East, and parts of South Asia; however, approximately 95% of malaria-related deaths occur in the African region. In 2022, global malaria cases rose up to 249 million, exceeding pre-pandemic levels, with 94% occurring in the WHO African Region. Nigeria, the Democratic Republic of Congo, Uganda, and Mozambique accounted for nearly half of all cases. Climate change and extreme weather events, such as the floods in Pakistan, contributed to outbreaks and resurgence in non-endemic regions. Malaria deaths reached 608,000, with over half concentrated in four African countries(3).

1.1.2 Disease: signs, symptoms, and diagnosis

Malaria symptoms are primarily caused by the circulation of the asexual stage of the parasite, mainly schizont stages and rupture of parasite-infected erythrocytes. The disease can present with

nonspecific symptoms, often resembling common viral infections, which may delay diagnosis. Fever, chills, and headache are the most common symptoms, with additional signs including dizziness, fatigue, abdominal pain, and mild gastrointestinal issues. Other physical findings may include tachycardia, jaundice, hepatomegaly, and splenomegaly(4).

1.1.3 Sever Malaria

Severe malaria is a life-threatening condition and one of the malignant forms of malaria, resulting from infection with *Plasmodium falciparum*. Most deaths occur within the first 24 hours of hospital admission. Sever malaria is characterized by hyperparasitemia and severe complications, such as cerebral malaria and severe anemia. The pathogenesis of severe malaria involves the sequestration of parasite-infected erythrocytes in the microvasculature and the formation of rosettes between infected and uninfected red blood cells (RBCs). These processes lead to impaired blood flow, organ dysfunction, and tissue hypoxia due to insufficient oxygen delivery.

This sequestration is mediated by variant surface antigens (VSAs) expressed on the surface of infected RBC (iRBCs). The primary VSAs implicated in severe malaria include PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1), RIFINs (Repetitive Interspersed Family), and STEVORs (Sub-Telomeric Open Reading Frame). The coordinated expression and antigenic variation of these VSAs enable the parasite to evade the host's immune system, leading to the development of vascular occlusion, which results in severe malaria(5).

1.2 *Plasmodium spp*

Malaria is caused by protozoan parasites of the genus Plasmodium, which infect a wide range of vertebrate hosts. Of the more than 200 recognized Plasmodium species, only five: *P.*

falciparum, P. vivax, *P. malariae, P. ovale*, and *P. knowlesi* are known to commonly infect humans. The first four species are primarily human-specific, whereas *P. knowlesi* is a zoonotic parasite naturally maintained in macaque monkeys in Southeast Asia. Transmission of the parasite occurs during the blood meal of an infected female *Anopheles* mosquito, which injects sporozoites from its salivary glands into the human host(6).

1.2.1 Biology and Life cycle

As illustrated in **(Figure 1.1)**, using the human host as an example, the life cycle of the *Plasmodium* parasite begins when an infected female *Anopheles* mosquito injects motile sporozoites into the skin during a blood meal. These sporozoites rapidly enter the bloodstream and migrate to the liver, where they invade hepatocytes and undergo an initial round of asexual replication known as exoerythrocytic schizogony. The resulting merozoites are released into the bloodstream and invade RBCs, initiating repeated asexual erythrocytic cycles that are responsible for the clinical manifestations of malaria. In some species, such as *P. vivax* and *P. ovale*, a proportion of liver-stage parasites persist as dormant forms called hypnozoites, which can reactivate weeks to months later, leading to malaria relapse. A subset of asexual blood-stage parasites differentiates into sexual forms (gametocytes), which are taken up by a mosquito during a subsequent blood meal. Within the mosquito midgut, male and female gametocytes fuse to form zygotes, which develop into motile ookinetes. These ookinetes traverse the midgut epithelium and mature into oocysts, within which thousands of sporozoites are generated. Upon rupture of the oocyst, sporozoites migrate to the mosquito's salivary glands, rendering the mosquito infectious and completing the life cycle (7), (8).

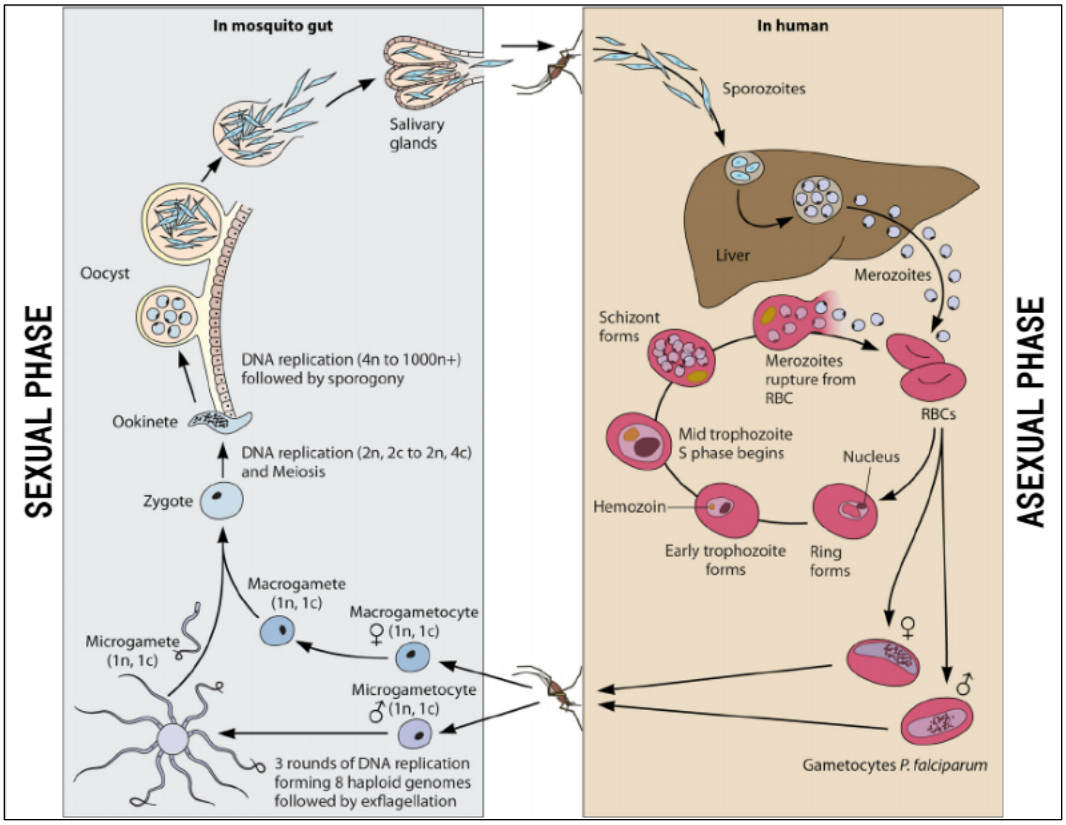

Figure 1.1 Asexual and sexual life cycle of *Plasmodium* parasite

1.2.2 *P. falciparum*

P. falciparum is the most virulent species of the human malaria parasites, responsible for the majority of severe and fatal malaria cases worldwide. It infects hundreds of millions annually and is particularly devastating in sub-Saharan Africa, where over 85% of global cases occur, especially among children under five years of age. According to the World Malaria Report 2023, malaria caused an estimated 608,000 deaths in 2022, with *P. falciparum* accounting for the overwhelming majority of these fatalities(3), (9).

P. falciparum is also associated with severe complications such as cerebral malaria, which affects the central nervous system and may result in long-term neurocognitive impairments in survivors, particularly in children(9). *P. falciparum* is also the primary causative agent of severe malaria

syndromes. Its virulence is largely attributed to three major surface-expressed protein families (RIFINs, STEVORs, and PfEMP1) which are exported by the parasite and expressed on the surface of iRBCs. These variant surface antigens mediate immune evasion and cytoadherence to endothelial receptors, contributing to microvascular sequestration, a hallmark of severe malaria pathogenesis(5).

1.3 *P. falciparum* variant surface antigens (VSA) and their role in sever malaria

The pathogenesis of *P. falciparum* is closely linked to its ability to evade the host immune system and adhere to host endothelial cells, largely mediated by variant surface antigens **(VSAs)** expressed on iRBCs as shown by white arrows in **(Figure 1.2)**. The three major VSA families includes: **PfEMP1**(*Plasmodium falciparum* Erythrocyte Membrane Protein 1), **STEVOR** (Subtelomeric Variable Open Reading frame), and **RIFIN** (Repetitive Interspersed Family Protein). VSA are encoded by multigene families and play distinct but complementary roles in immune evasion, cytoadhesion during the pathogenesis of sever malaria syndrom.

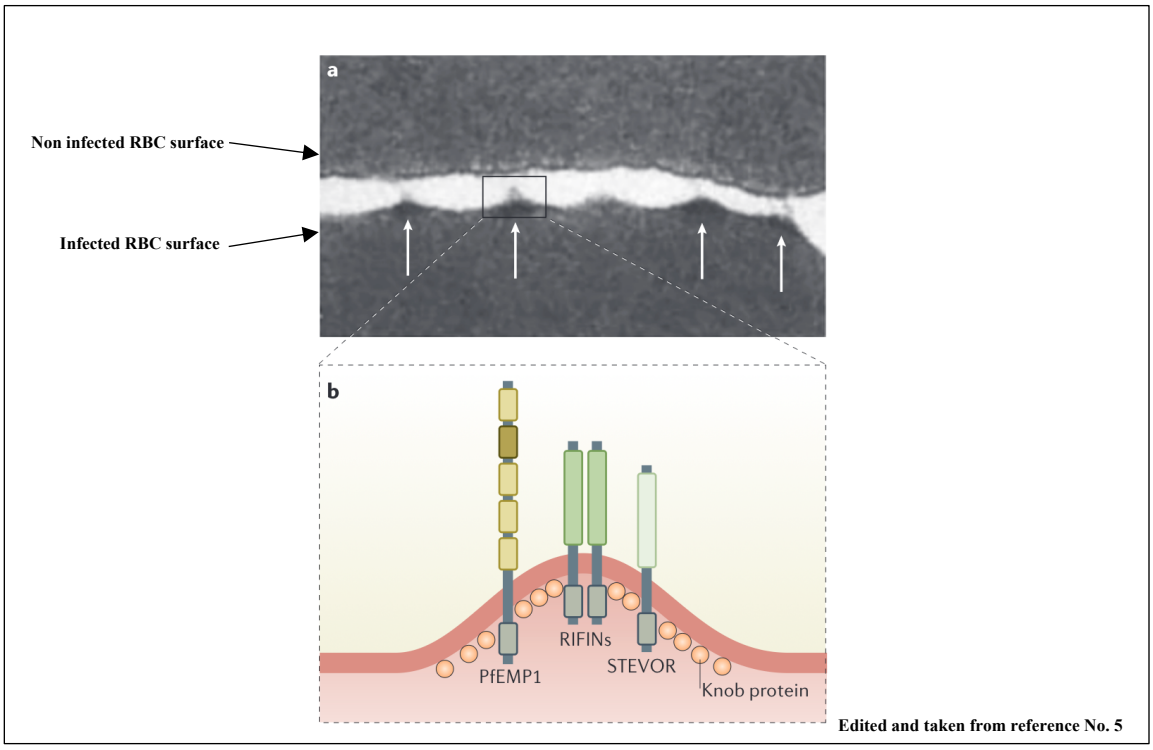

Figure 1.2 *P. falciparum* variant surface antigens (VSA) expressed on surface of infected RBCs

PfEMP1 is a major VSA that binds to various host receptors, including CD36, ICAM1, EPCR, chondroitin sulfate A, and heparan sulfate, on the surface of vascular endothelial cells(5). This interaction facilitates the sequestration of parasites within blood vessels. In the brain, such sequestration leads to severe neurological dysfunction, often accompanied by coma, and is frequently fatal(10). Another major VSA, repetitive interspaced family protein (RIFIN) interacts with several immunosuppressive receptors, such as LILRB1, LILRB2, and LAIR, which are expressed on a wide range of immune cells, including macrophages, T cells, NK cells, and B cells(11), (12). By engaging these receptors, specific RIFIN variants inhibit immune cell function, thereby enabling the parasite to evade host immune responses. The subtelomeric variable open reading frame (STEVOR) is also a member of VSA family and binds to glycoprotein C on the surface of RBCs (13). Through this interaction, infected RBCs form clumps with uninfected RBCs, leading to vascular occlusion and RBC sequestration, as shown in **(Figure 1.3).**

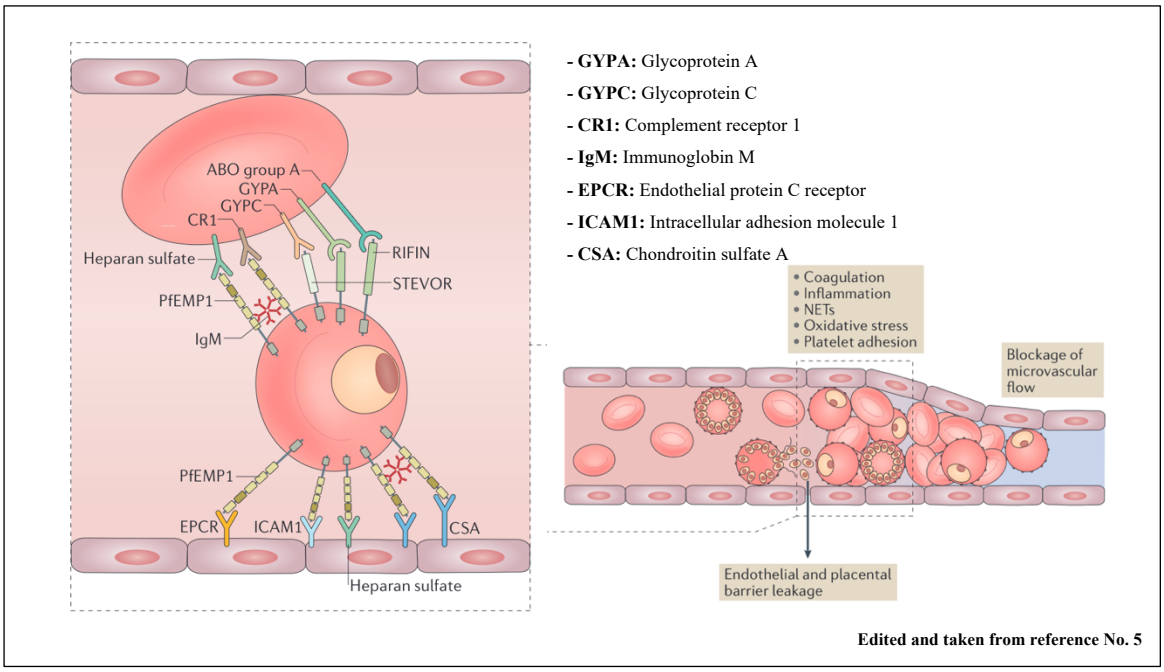

Figure 1.3 Model of sequestration of *P. falciparum* infected RBC during sever malaria

1.4 Subtelomeric and telomeric heterochromatin in *P. falciparum*

The *P. falciparum* genome (22.8 Mb) is distributed across 14 chromosomes. Each chromosome contains conserved central regions harboring essential housekeeping genes, and polymorphic subtelomeric domains that cluster variant antigen-encoding gene families (*var*, *rif*, *stevor*), which encode virulence surface antigens (VSAs). These subtelomeric regions support antigenic variation and immune evasion. Chromosome ends feature tandem telomeric repeats (GGGTTT/CA), followed by subtelomeric noncoding known as telomere-associated (TAS), which comprises species-specific coding and noncoding elements. The noncoding portion consists of six ordered repetitive blocks, termed telomere-associated repetitive elements (TAREs), comprises about 20–40 kb **(Figure 1.4)**. Although TAREs vary in size and sequence, their relative order is conserved across chromosomes, playing a crucial role in regulating virulence gene expression and genome stability(14).

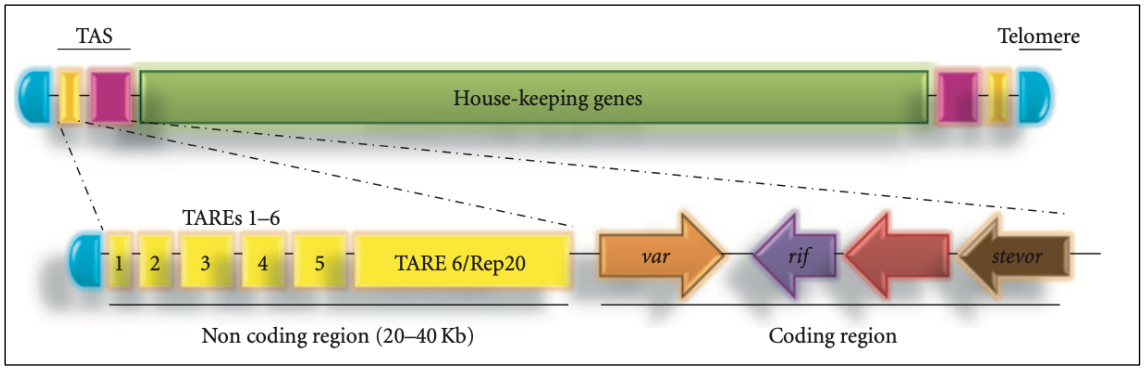

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Figure 1.4 A model of *P. falciparum* chromosome organization

1.4.1 Role of the subtelomeric region in *P. falciparum* virulence

PfEMP1, RIFIN, and STEVOR are encoded by multi-gene families comprising approximately 60, 150, and 30 genes, respectively. The sequences of these gene family members exhibit a high degree of diversification(13). Approximately 62.3 % of the PfEMP1, 95.5 % of RIFIN, and 96.9 % of STEVOR genes, referred to as *var*, *rif*, and *stevor*, are tandemly located in the subtelomeric

regions, which are proximal to the telomere ends. ChIP (Chromatin Immunoprecipitation) -on-chip experiments have demonstrated that lysine 9 of histone H3 in the subtelomeric regions is tri- and di-methylated, indicating heterochromatin formation in these chromosomal regions(15). Due to this heterochromatin formation, the expression of *var*, *rif*, and *stevor* genes is usually suppressed. However, a small number of *var*, *rif*, and *stevor* loci are randomly derepressed by an unknown mechanism, leading to their expression. As a result, the parasite expresses only a limited number of PfEMP1, RIFIN, and STEVOR proteins on the surface of iRBCs, and this expression is maintained epigenetically. When the host acquires antibodies against the expressed PfEMP1, RIFIN, and STEVOR proteins, the parasite naturally switches to expressing different members of these gene families, thereby evading immune recognition(16).

1.4.2 Telomere healing

When a double-strand break occurs within the subtelomeric region of *P. falciparum*, the cleavage site is likely recognized by a putative protein complex containing an endonuclease, initiating DNA resection away from the telomere. This process generates a single-stranded 3' overhang, which, upon encountering a complementary sequence to the template RNA of telomerase, allows the telomerase complex to bind and directly add telomere repeats at the recognized site. This repairment of the double-strand break by telomerase is called as “telomere healing” and frequently found in the chromosome ends of laboratory-maintained *P. falciparum* strains **(Figure 1.5)**. In some strains, 85–110 kb of the subtelomeric region is truncated from the original chromosome ends, resulting in the loss of all VSA genes including *var*, *rif*, and *stevor* on these chromosome arms(17).

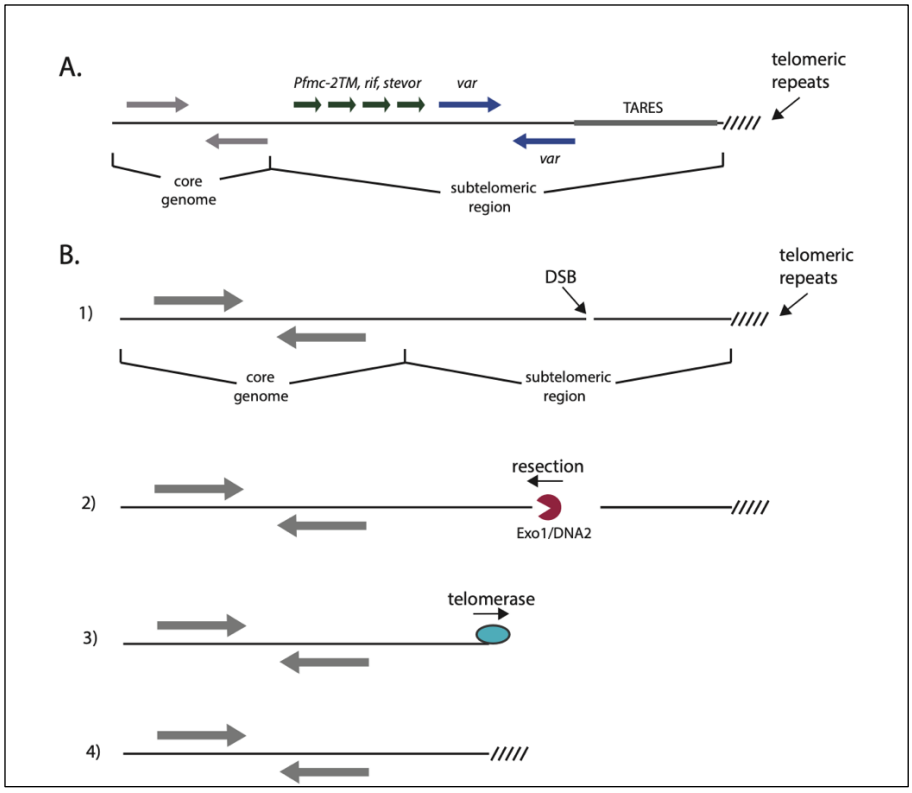

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Figure 1.5 Repair of double strand breaks within subtelomeric chromosomal regions **(A)** Chromosome structure **(B)** Telomere healing process

1.5 CRISPR-associated Cas proteins as tool for genome editing

Genome editing in *P. falciparum* has traditionally been difficult due to inefficient and laborious methods. While zinc-finger nucleases allow targeted DNA modification, their complex design and high cost limit their use. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas system, a prokaryotic immune mechanism, has emerged as a simpler and more efficient alternative. This tool enables precise, cost-effective genome editing in *P. falciparum*(18).

1.5.1 Use of CRISPR/Cas9 for *Plasmodium* genome editing

The CRISPR-Cas9 system comprises two main components: the Cas9 endonuclease (derived from

Streptococcus pyogenes) and a single-guide RNA (sgRNA). The sgRNA directs Cas9 to a specific DNA sequence, where the endonuclease introduces a double-strand break (DSB) typically 3 base pairs upstream of a protospacer adjacent motif (PAM) sequence, usually "NGG". Following cleavage, the host cell repairs the DSB via either the error-prone non-homologous end-joining (NHEJ) pathway, which can result in insertions or deletions (indels), or the more accurate homology-directed repair (HDR) pathway when a donor template is available **(Figure 1.6).** In Plasmodium species, which lack a canonical NHEJ pathway, HDR is the primary mechanism for DNA repair(19), (20).

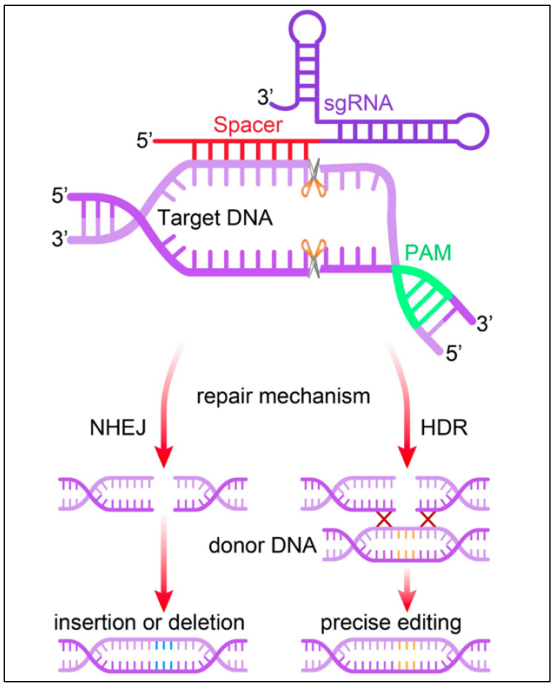

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Figure 1.6 Mechanism of the CRISPR/Cas9 gene editing system

Cas9 activity is significantly reduced in heterochromatic regions due to limited DNA accessibility, particularly at nucleosome-bound or densely methylated sites. In contrast, euchromatin allows more efficient Cas9 binding and cleavage, highlighting the importance of chromatin state in genome editing outcomes(21), (22).

1.5.2 CRISPR/Cas12a:

Cas12a (also known as Cpf1) is a type V CRISPR-associated endonuclease recognized for its broad utility in genome editing. It was originally identified in *Acidaminococcus* and *Lachnospiraceae* species and is distinguished by its ability to facilitate simplified and multiplex genome editing. Cas12a is guided by a single crRNA and does not require a tracrRNA. Its mechanism **(Figure 1.7)** involves the formation of a crRNA-Cas12a complex that targets DNA sequences adjacent to a thymine-rich protospacer adjacent motif (PAM), typically TTTV (where V = A, C, or G). Upon binding, the crRNA spacer sequence (approximately 21 nucleotides) hybridizes with the complementary DNA strand, forming an R-loop structure. This conformational change activates the RuvC and nuclease (NUC) domains of Cas12a, which generate a double-strand break (DSB) with sticky ends. The unique ability of Cas12a to process crRNAs and induce precise cuts makes it a powerful tool for genome editing(23).

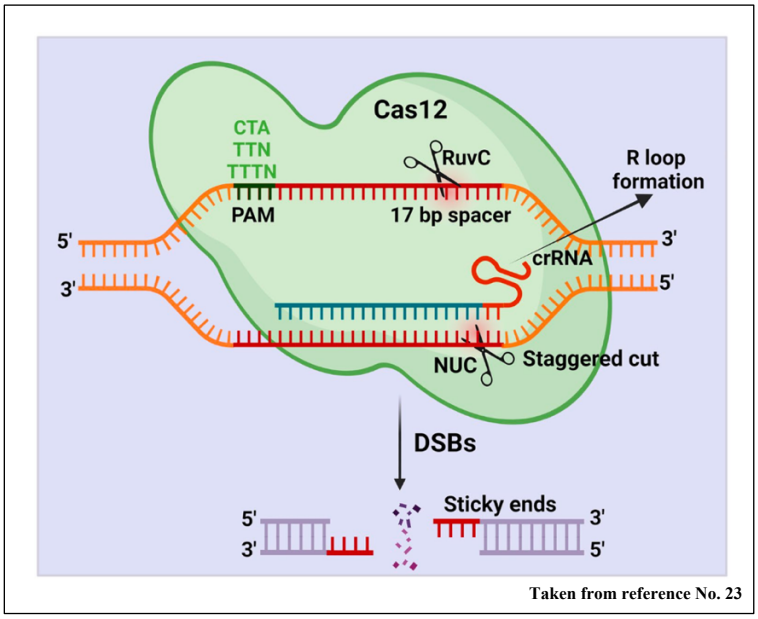

Figure 1.7 Schematic illustration of CRISPR/Cas12 mechanism

While CRISPR/Cas9 has been extensively applied in *Plasmodium* research, the Cas12a (Cpf1)

system has recently emerged as a promising alternative due to its T-rich protospacer adjacent motif (PAM) requirement, which aligns with the AT-rich nature of the *Plasmodium* genome. Although experimental applications of Cas12a in Plasmodium gene editing are still limited, its diagnostic utility has already been demonstrated in highly sensitive and diagnostic detection assays. These characteristics make Cas12a a potential tool for both functional genomic studies and molecular surveillance in malaria research(24), (25).

1.5.3. CRISPR/Cas12 Ultra

Cas12a Ultra is an engineered variant of the *Acidaminococcus*-derived Cas12a (AsCas12a) nuclease, developed to address the limitations of the wild-type enzyme in genome editing. It incorporates two key amino acid substitutions: M537R (methionine to arginine) and F870L (phenylalanine to leucine), which enhance DNA-binding affinity and catalytic efficiency, respectively. These amino acid modifications significantly improve genome editing performance, achieving editing efficiencies approaching 100% at multiple clinically relevant loci in different types of human primary cells. Cas12a Ultra also supports efficient multiplex gene editing and targeted gene knock-ins, such as the insertion of chimeric antigen receptor (CAR) constructs into natural killer (NK) cells to enhance anti-tumor responses. These characteristics make Cas12a Ultra a promising tool for next-generation allogeneic cell therapies, particularly in oncology and regenerative medicine fields(26).

To date, no published studies have reported the use of Cas12a Ultra for genome editing in *Plasmodium* species.

1.6 The objective of this study:

VSAs of *P. falciparum* are encoded by large multigene families comprising dozens to hundreds of genes, primarily located in heterochromatic regions such as subtelomeric regions that are notoriously resistant to genetic manipulation. Moreover, due to the parasite's ability to undergo antigenic variation by randomly switching VSA expression through poorly understood epigenetic mechanisms, it is challenging to utilize parasites that clearly express specific target VSAs for experimental purposes. As a result, functional characterization of these antigens has been limited, despite their well-established clinical significance in malaria pathology. To overcome these challenges, I propose a genetic strategy involving the deletion of all VSA genes, followed by the reintroduction of specific target VSA genes, followed by the targeted reintroduction of specific VSA genes. For this purpose, I developed a method for deleting VSA genes located in subtelomeric regions by combining the heterochromatin-accessible AsCas12a-Ultra nuclease with telomere healing. I selected the engineered AsCas12a-Ultra variant due to its enhanced nuclease activity compared to the wild-type AsCas12a. Additionally, AsCas12a is well suited for multiplex gene editing, as it possesses RNase activity that enables processing of tandemly arrayed crRNAs into individual functional units. In conclusion, the present method offers a powerful approach for generating null-VSA parasites, generating null-VSA parasites, which can serve as recipient parasites for transfection experiments aimed at the functional analysis of PfEMP1, RIFIN, and STEVOR.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Cultivation and Synchronization of the Parasite

The *Plasmodium falciparum* 3D7 strain was sourced from the Malaria Research and Reference Reagent Resource Center (MR4, http://www.mr4.org). Parasite cultures were maintained in type O human erythrocytes (hematocrit 2%) obtained from the Japanese Red Cross Aichi Blood Centre. Cultivation was performed using a complete culture medium composed of RPMI-1640 supplemented with 10% human serum (Japanese Red Cross Aichi Blood Centre), 10% AlbuMAX I (GIBCO BRL), 25 mM HEPES, 0.225% sodium bicarbonate, 0.38 mM hypoxanthine, and 10 µg/mL gentamicin. Cultures were incubated under reduced oxygen conditions (90% N_2, 5% CO_2, and 5% O_2). When parasitemia reached approximately 5%, infected erythrocytes were transferred into fresh uninfected erythrocytes to continue propagation. Parasite synchronization was performed using a 5% D-sorbitol treatment following a standard protocol (Lambros & Vanderberg, 1979). In brief, infected red blood cells were rapidly mixed with ten volumes of 5% D-sorbitol solution and incubated at 37 °C for 8 minutes. Post-incubation, cells were harvested by centrifugation, washed in incomplete medium (RPMI-1640 with 25 mM HEPES, 0.225% sodium bicarbonate, 0.38 mM hypoxanthine, and 10 µg/mL gentamicin), and subsequently returned to complete medium for continued culture as previously described (27) (28).

2.2 Preparation of AsCas12 ultra

The *Acidaminococcus sp*. Cas12a derivative nuclease (AsCas12a-Ultra) was generated from the wild-type AsCas12a through site-directed mutagenesis. Two oligonucleotides were designed: Sense-Cas12-S (5′-GTGCAAAAGCTAGCGCCCCAAAGAAGAAGCGGAAGGTCGGTATCC-3′) and Anti-sense Cas12-AS (5′-CATCACCGCTCGAGGTCGACAAAATACCAATAATACC-3′). These primers were used in a PCR reaction to amplify the region containing the desired point mutations,

where methionine at position 537 (Met537) and phenylalanine at position 870 (Phe870) were substituted with arginine (Arg) and leucine (Leu), respectively. The wild-type AsCas12a DNA template was obtained from Addgene. PCR amplification was carried out under standard conditions, and the resulting products were verified by agarose gel electrophoresis (LONZA). The PCR fragments were then purified using the Wizard® DNA Purification Kit (Promega). Successful introduction of the mutations was confirmed by Sanger sequencing.

2.3 Plasmid construction

All cloning procedures were performed by amplifying the targeted DNA sequences from template targeted DNA using specific primers and PrimeSTAR GXL DNA Polymerase (Takara Bio, Japan). The PCR products were subsequently cloned into the appropriate plasmid vectors at designated sites using the Seamless Ligation Cloning Extract (SLiCE) method, as described previously(29). The resulting recombinant plasmids were generated within *Escherichia coli* DH5α host, and sequence accuracy was confirmed using Sanger sequencing.

To generate the base vector expressing the AsCas12 Ultra enzyme, referred to as pCas12-UL, the DNA backbone vector pBluescriptSK(+), which contains PfCent5, the 3′ UTR of Pbdhfr-ts, and the Pbef1α promoter, was linearized by inverse PCR. The *hdfr-yFCU* cassette was subsequently cloned downstream of the Pbef1α promoter. The resulting vector was then linearized again by PCR, and a purified DNA fragment encoding the mutated AsCas12a enzyme (AsCas12Ultra), prepared as previously described in Section 2.1, was cloned into it. Each designated crRNA was cloned into *BsmB1* site on pCas12-UL base plasmids , to result in new plasmids addressed as in **Table 2.9**.

2.4 CRISPR RNA (crRNA) design:

Candidate crRNAs were designed using the CRISPOR program (https://crispor.gi.ucsc.edu/), and the most suitable sequences were selected based on predicted cleavage efficiency and the number of potential off-target sites. The selected crRNA sequences were synthesized along with direct repeat (DR) sequences by PCR amplification using PrimeStar GXL DNA polymerase (Takara) and oligonucleotides listed in **Table 2.10.** The resulting PCR products were confirmed by agarose gel electrophoresis (LONZA) and subsequently purified using the Wizard DNA Purification Kit (Promega), and cloned into target plasmids.

2.5 Transfection of *P. falciparum* and selection for transgein parasites

The transfection of the parasites was performed by the invasion of DNA-loaded erythrocytes(30). Briefly, RBCs were washed three times with incomplete medium and resuspended to 50% hematocrit. All plasmids were purified using the QIAGEN miniprep kit (QIAGEN), precipitated with ethanol, and dissolved in incomplete cytomix (120 mM KCl, 0.15mM CaCl2, 2mM EGTA, 5mM MgCl2, 10mM K2HPO4/ KH2PO4, 25 mM HEPES, pH 7.6). The washed RBCs and 12.5 mg of the DNA samples were combined in an ice-cold cuvette and then electroporated using a Bio-Rad Gene Pulser II at 0.31 kV and 950 mF. The DNA-loaded RBCs were immediately washed three times with complete medium and then infected with the parasites. The medium was changed every 24 hours, and the drug selection of the transgenic parasites using pyrimethamine (2.5 ng/ml), followed by 5-fluoro-cytosine (5-FC) was initiated 4 days after the electroporation(28), to remove the introduced plasmids.

2.6 Purification of genomic DNA

Purification of genomic DNA was perfrormed as mentioned previousely with some modifications(31),(32) . The genomic DNA (gDNA) was isolated from *P. falciparum*-infected red blood cells using a small-scale phenol–chloroform-based extraction protocol. Infected red blood cells were first lysed using a red blood cell lysis buffer consisting of 1.5 M ammonium chloride (NH_4Cl), 0.1 M potassium bicarbonate ($KHCO_3$), and 0.01 M ethylenediaminetetraacetic acid (EDTA). The resulting parasite pellet was resuspended in HNE buffer containing 10 mM Tris-HCl, 150 mM NaCl, and 10 mM EDTA (pH 8.0), supplemented with 0.1% sodium dodecyl sulfate (SDS).

Protein digestion was carried out by adding proteinase K to a final concentration of 40 µg/mL, followed by incubation until complete lysis was achieved. After digestion, gDNA was extracted using a phenol/chloroform/isoamyl alcohol (PCI, 25:24:1) mixture and subsequently precipitated with cold ethanol. The DNA pellet was air-dried and resuspended in nuclease-free water or TE buffer. DNA purity and concentration were determined using the Qubit fluorometric quantification system (Invitrogen).

2.7 Quantitative (qPCR)

To assess genome cleavage efficiency, quantitative PCR (qPCR) was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) on a QuantStudio™ Real-Time PCR System. Genomic DNA was extracted from parasites was used as template for reaction. Each 20 µL qPCR reaction contained 10 µL of SYBR Green master mix, 0.5 µL each of forward and reverse primers (10 µM), 2 µL of DNA (10–20 ng), and 7 µL of nuclease-free water. Primers used for this reactions are listed in **Table 2.11** . The cycling protocol consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, ending with a melt curve stage (60–95°C). Relative cleavage efficiency was calculated using the ΔΔCt method, normalized to the

reference amplicon and compared to the wild-type control, using the formula: (1 – 2^–ΔΔCt) × 100%. All reactions were run in technical triplicates and biological duplicates. Data analysis and Ct determination were conducted using QuantStudio™ Design and Analysis Software v1.5.1.

2.8 Sequence analysis of subtelomeric region deleted parasites

To confirm the deletion of the targeted subtelomeric regions and the formation of de novo telomeres, long-read sequencing was performed with minor modifications to a previously described protocol(20). A total of 2.5 µg of high-quality genomic DNA was used for library preparation using the Ligation Sequencing Kit(Oxford Nanopore Technologies), following the manufacturer's instructions. Sequencing was conducted on a MinIONdevice (Oxford Nanopore Technologies). The resulting long reads were mapped to the *Plasmodium falciparum* 3D7 reference genome available in PlasmoDB (https://plasmodb.org). To investigate de novo telomere formation at the cleavage sites, a hypothetical modified genome was generated by manually adding telomeric repeat sequences to the expected cleavage regions. The long-read data were then remapped to this modified genome.Reads aligned seamlessly to the newly introduced telomeric regions, indicating the successful addition and extension of telomeric repeats at the CRISPR/Cas-induced breakpoints.

Table 2.9 crRNA sequences used in this study

CrRNA Sequence (5`to 3') cloned into linearized pCas12-UL	Resulted plasmid name
AATTTCTACTCTTGTAGAT TGAACCTATGTCAACGATAGA AATTTCTACTCTTGTAGAT	pCas12-UL2-1
AATTTCTACTCTTGTAGAT GAGGTGTAGGTATATGTACAT AATTTCTACTCTTGTAGAT	pCas12-UL2-2
AATTTCTACTCTTGTAGATTGAACCTATGTCAACGATAGAAATTTCTACTCTTGTAGATG AGGTGTAGGTATATGTACATAATTTCTACTCTTGTAGAT	pCas12-UL2-1/2
AATTTCTACTCTTGTAGAT AGTACCTTCTCTTACAGCTGG	pCas12-UL 7crrna
AATTTCTACTCTTGTAGAT TGTAGTAGGTATGGTACACCA	
AATTTCTACTCTTGTAGAT CACACCGAATGGCAAAAATGA	
AATTTCTACTCTTGTAGAT CGCAGCAGAGGCTACAAGTGT	
AATTTCTACTCTTGTAGAT CAGACTTATATAACTGCTTCT	
AATTTCTACTCTTGTAGAT TTCAACAAATACCACATTACT	
AATTTCTACTCTTGTAGAT CACCAAATACCAGGTTATTATAATTTCTACTCTTGTAGAT	

- Blue colored sequences indicates direct repeats (DR) sequences.

Table 2.10 Oligonucleotides used for crRNA generation in this study

Primer name	Sequence	Targeted gene
CR-1	AATTTCTACTCTTGTAGATTGAACCTATGTCAACGATAGAAATTTCTACTCTTGTAGAT	**PF3D7_0201300**
CR-2	ATCTACAAGAGTAGAAATTTCTATCGTTGACATAGGTTCAATCTACAAGAGTAGAAATT	
CR-3	AATTTCTACTCTTGTAGATGAGGTGTAGGTATATGTACATAATTTCTACTCTTGTAGAT	**PF3D7_0221400**
CR-4	ATCTACAAGAGTAGAAATTATGTACATATACCTACACCTCATCTACAAGAGTAGAAATT	
CR-5	AATTTCTACTCTTGTAGATAGTACCTTCTCTTACAGCTGG	**PF3D7_0532900**
CR-6	CCAGCTGTAAGAGAAGGTACTATCTACAAGAGTAGAAATT	
CR-7	AATTTCTACTCTTGTAGATTGTAGTAGGTATGGTACACCA	**PF3D7_1200600**
CR-8	TGGTGTACCATACCTACTACAATCTACAAGAGTAGAAATT	
CR-9	AATTTCTACTCTTGTAGATCACACCGAATGGCAAAAATGA	**PF3D7_1479400**
CR-10	TCATTTTTGCCATTCGGTGTGATCTACAAGAGTAGAAATT	
CR-11	AATTTCTACTCTTGTAGATCGCAGCAGAGGCTACAAGTGT	**PF3D7_1149800**
CR-12	ACACTTGTAGCCTCTGCTGCGATCTACAAGAGTAGAAATT	
CR-13	AATTTCTACTCTTGTAGATCAGACTTATATAACTGCTTCT	**PF3D7_0937300**
CR-14	AGAAGCAGTTATATAAGTCTGATCTACAAGAGTAGAAATT	
CR-15	AATTTCTACTCTTGTAGATTTCAACAAATACCACATTACT	**PF3D7_1000600**
CR-16	AGTAATGTGGTATTTGTTGAAATCTACAAGAGTAGAAATT	
CR-17	AATTTCTACTCTTGTAGATCACCAAATACCAGGTTATTATAATTTCTACTCTTGTAGAT	**PF3D7_1101300**
CR-18	ATCTACAAGAGTAGAAATTATAATAACCTGGTATTTGGTGATCTACAAGAGTAGAAATT	

Table 2.11 Oligonucleotides used for genotyping and qPCR in this study

Primer name	Sequence (5`to 3')	Target
P1	ATGTGCGCTACAAGAAGCTG	PF3D7_0200100 Forward
P2	TTGATCTCCCCATTCAGTCA	PF3D7_0200100 Reverse
P3	CAATTTTGGGTGTGGAATCA	PF3D7_0223500 Forward
P4	CACTGGCCACCAAGTGTATC	PF3D7_0223500 Reverse
P5	AAGAAAGTGCCACAACATGC	PF3D7_0533100 Forward
P6	GTTCGTACGCCTGTCGTTTA	PF3D7_0533100 Reverse
P7	CAAGAAATGGGTATATCAACTCTACGTGG	PF3D7_1479600 Forward
P8	CCTTGAGTCATCCGTTGAACTTTCAATGC	PF3D7_1479600 Reverse
P9	TCGATTATGTGCCGCAGTAT	PF3D7_1200400 Forward
P10	TTCCCGTACAATCGTATCCA	PF3D7_1200400 Reverse
P11	TGCTGAAGACCAAATTGAG	PF3D7_1150400 Forward
P12	TTGTTGTGGTGGTTGTTGTG	PF3D7_1150400 Reverse
P13	TGACCAAGACGAAGTATGGAA	PF3D7_0937600 Forward
P14	TTGATCTCTGTTCGCTGTCC	PF3D7_0937600 Reverse
P15	GACGAGGAGTCGGAAAAGAC	PF3D7_1000100 Forward
P16	TGGACAGGCTTGTTTGAGAG	PF3D7_1000100 Reverse
P17	GACGGCTACCACAGAGACAA	PF3D7_1100200 Forward
P18	CGTCATCATCGTCTTCGTTT	PF3D7_1100200 Reverse

CHAPTER 3 – RESULTS

3.1 Development of a stategy for deletion of VSA genes

Studies on VSAs have typically been conducted using clonal parasite lines that express specific VSAs capable of binding to host receptors(33), (34) . However, the expression of the target VSA can be altered or lost due to switching of expression patterns and the natural truncation of subtelomeric regions following double-strand breaks. Moreover, heterochromatin formation and the extensive number of VSA gene copies have posed significant obstacles to the genetic manipulation of these loci. Thus, despite their clinical significance in malaria pathology, the biological functions of VSAs remain incompletely understood, owing to these associated experimental challenges. To overcome these challenges, I propose a strategy (**Fig. 3-A**) involving the deletion of all VSA genes, followed by the reintroduction of specific target VSA genes., I developed a genome editing method for the simultaneous deletion of multiple VSA genes as an initial step. Specifically, I attempted to delete VSA genes by artificially inducing double-strand breaks at subtelomeric regions by AsCas12-Ultra, followed by repair of the broken sites through telomere healing, a phenomenon in which double-strand breaks near telomeres are repaired by *de novo* telomere addition mediated through action of telomerase enzyme, and this process is occasionally observed in *P. falciparum*.

3.2 Successful construction of AsCas12-Ultra expressing vectors

To apply the subtelomeric region removal strategy, I employed the *Acidaminococcus sp.* Cas12a (AsCas12a) nuclease to introduce double-strand breaks at subtelomeric regions, as it has been shown to localize to phase-separated nucleosomes, which are characteristic of heterochromatic regions, *in vitro*. I used the engineered variant AsCas12a-Ul which is characterized by its ability of multiplex gene editing through processing of tandemly arrayed crRNAs into individual units using its own RNase activity.

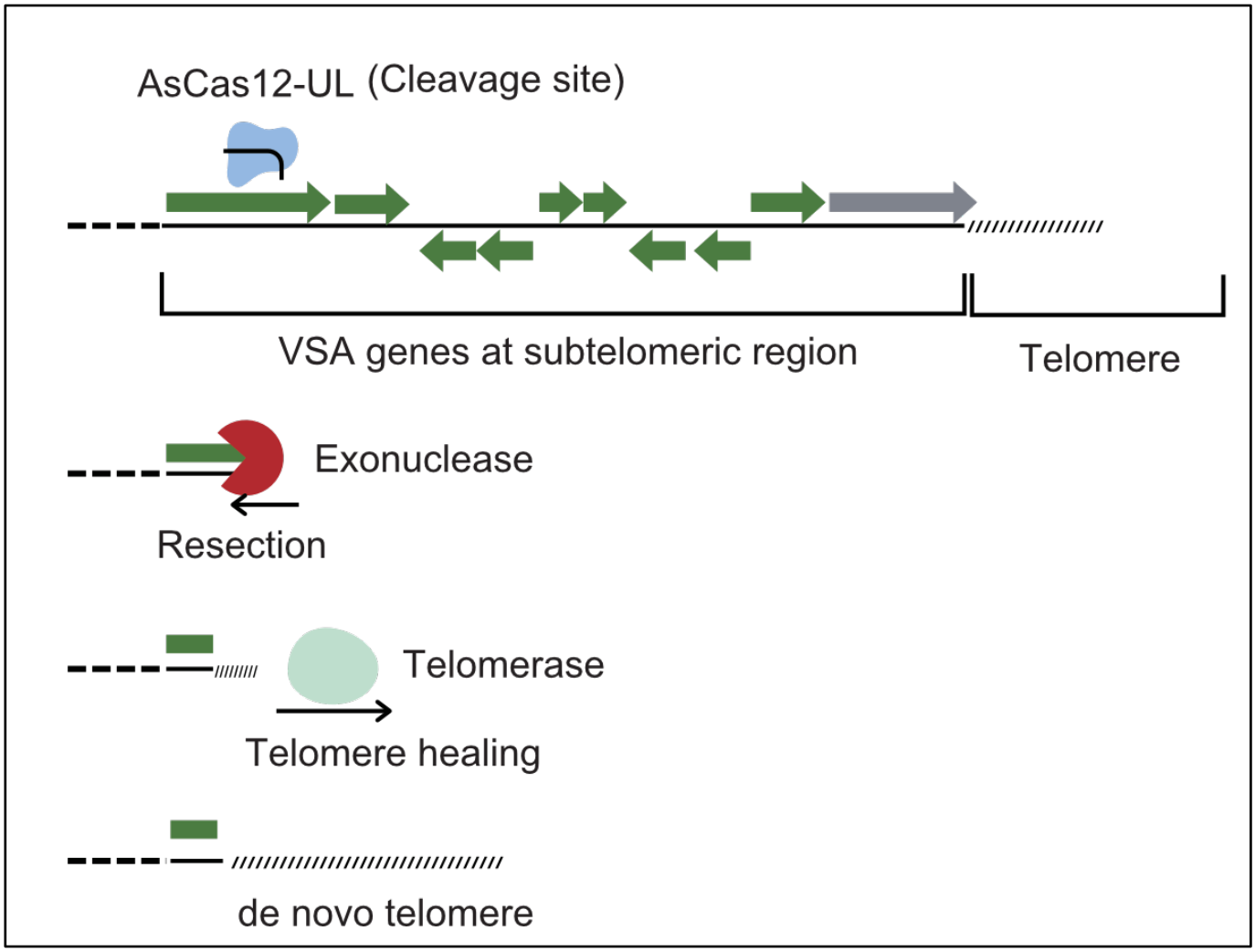

Figure. 3-A: Developed strategy to remove VSA genes at subtelomeric region of *P. falciparaum*

The crRNA candidates were selected based on predicted cleavage efficiency and the number of potential off-target sites. A base plasmid expressing *AsCas12a-ultra* gene nuclease enzyme, designated as pCas12-UL was constructed (**Fig. 3-B**). Each targeted gene crRNA was cloned into pCas12-UL. To enable selection of transgenic parasites, we incorporated the *human dihydrofolate reductase* (*hdhfr*) gene and the *yFCU* fusion gene, which comprise *yeast cytosine deaminase* and *uridyl phosphoribosyltransferase* as positive and negative selection markers, respectively. These were further fused into a single *hdhfr-yFCU* gene, expressed as a fusion protein. The *hdhfr* confer pyrimethamine resistance to the parasites, and the parasites having *yFCU* can be killed specifically

by treating with 5-fluoro-cytosine (5-FC). Both *AsCas12a-ultra* and *hdhfr-yFCU* genes were transcribed under the control of the *P. berghei* elongation factor promoter a, which enables simultaneous, bidirectional transcription of two genes. The crRNA, along with the essential direct repeat (DR) sequence required for crRNA-AsCas12a complex formation, was transcribed under the control of the *P. falciparum* U6 promoter.

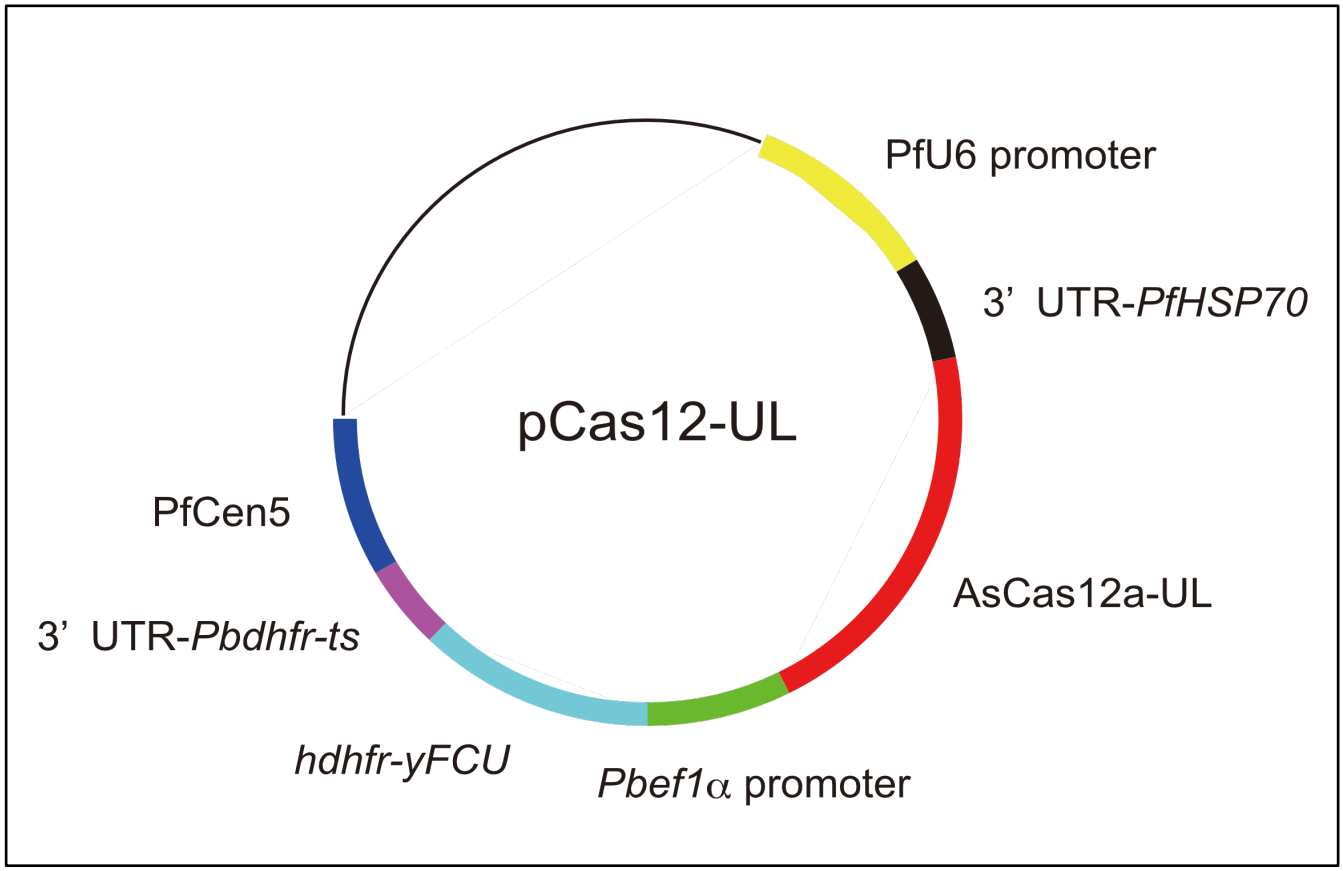

Figure. 3-B: Map of the pCas12-UL plasmid Map of the pCas12-UL plasmid

3.3 Assessment of AsCas12a-Ultra activity in removing *rif* and *stevor* genes in chromosome 2

To examine whether the subtelomeric region could be removed using pCas12-UL, I targeted PF3D7_0201300 and PF3D7_0221400 on chromosome 2 **(Fig. 3-C)**, which code for encode RIFIN and STEVOR, respectively. The plasmids with crRNA that target PF3D7_0201300 and PF3D7_0221400 were constructed and named pCas12-UL-chr2-1 and -chr2-2, respectively. Also, to

whether PF3D7_0201300 and PF3D7_0221400 could be cleaved simultaneously, a tandemly linked crRNA that contains sequences

Figure. 3-C: Schematic representation of chromosome 2. Target genes for *AsCas12a-UL* are shown in red, while genes used for genotyping PCR are shown in green.

that targets the two genes, was constructed and named pCas12-UL-chr2-1/2. *P. falciparum* 3D7 strain was transfected with 5 µg of pCas12-UL-chr2-1 and -chr2-2, and chr2-1/2, followed by selecting transgenic parasites using pyrimethamine(20). Subsequently, to remove the introduced plasmids, the selected parasites were further treated with 5-FC. The resultant transgenic parasites with pCas12-UL-chr2-1 and -chr2-2, and chr2-1/2 were named subdel-chr2-1 and -chr2-2, and chr2-1/2.

3.4 PCR confirms the deletion of *rif* and *stevor* genes at chromosome 2

To detect the deletion of subtelomeric regions on chromosome 2, I performed genotyping PCR of transgenic parasites using primer sets specific to *PF3D7_0200100* and *PF3D7_0223500***.** These two genes would be lost from transgenic parasites, if the subtelomeric regions were able to be removed. Electrophoresis of the PCR products revealed weaker signal intensities in subdel-chr2-1, subdel-chr2-2, and chr2-1/2 compared to the parental *P. falciparum* 3D7 strain, suggesting the deletion of subtelomeric regions at each end and both ends of chromosome 2 **(Fig. 3-D)**.

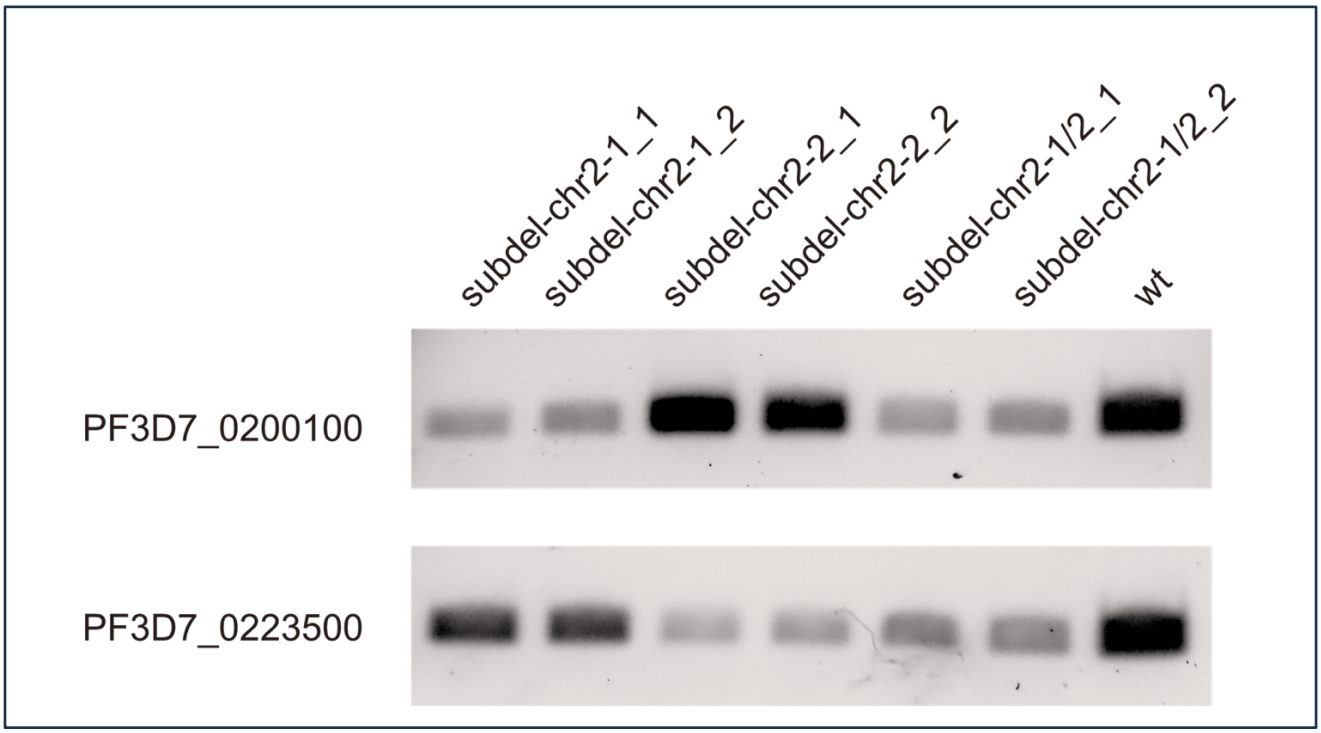

Figure. 3-D: Genotyping PCR. PCR was performed for transgenic parasites obtained by two independent transfection experiments. *PF3D7_0200100* and *PF3D7_0223500* were the target loci for this experiment.

Quantitative PCR analysis using similar primer sets supported the deletion of subtelomeric regions, and the cleavage efficiencies of crRNAs for PF3D7_0201300 and PF3D7_0221400 were estimated as more than 99 % **(Fig. 3-E)**. However, unexpected cleavage events by the AsCas12a-ultra–PF3D7_0221400_crRNA and –PF3D7_0201300_crRNA complexes were observed in subdel-chr2-1 and subdel-chr2-2, respectively, with cleavage efficiencies of approximately 20–40% **(Fig. 3-E)**. Since the VSA sequences are similar to each other, it is presumed that unexpected off-target cleavage is likely to occur.

3.5 Long read sequencing confirms deletion of *rif* and *stevor* genes at chromosome 2

Clonal parasite lines were established from subdel-chr2-1/2 and synbmitted for with long read sequencer MinION (Oxford nanopore technology).

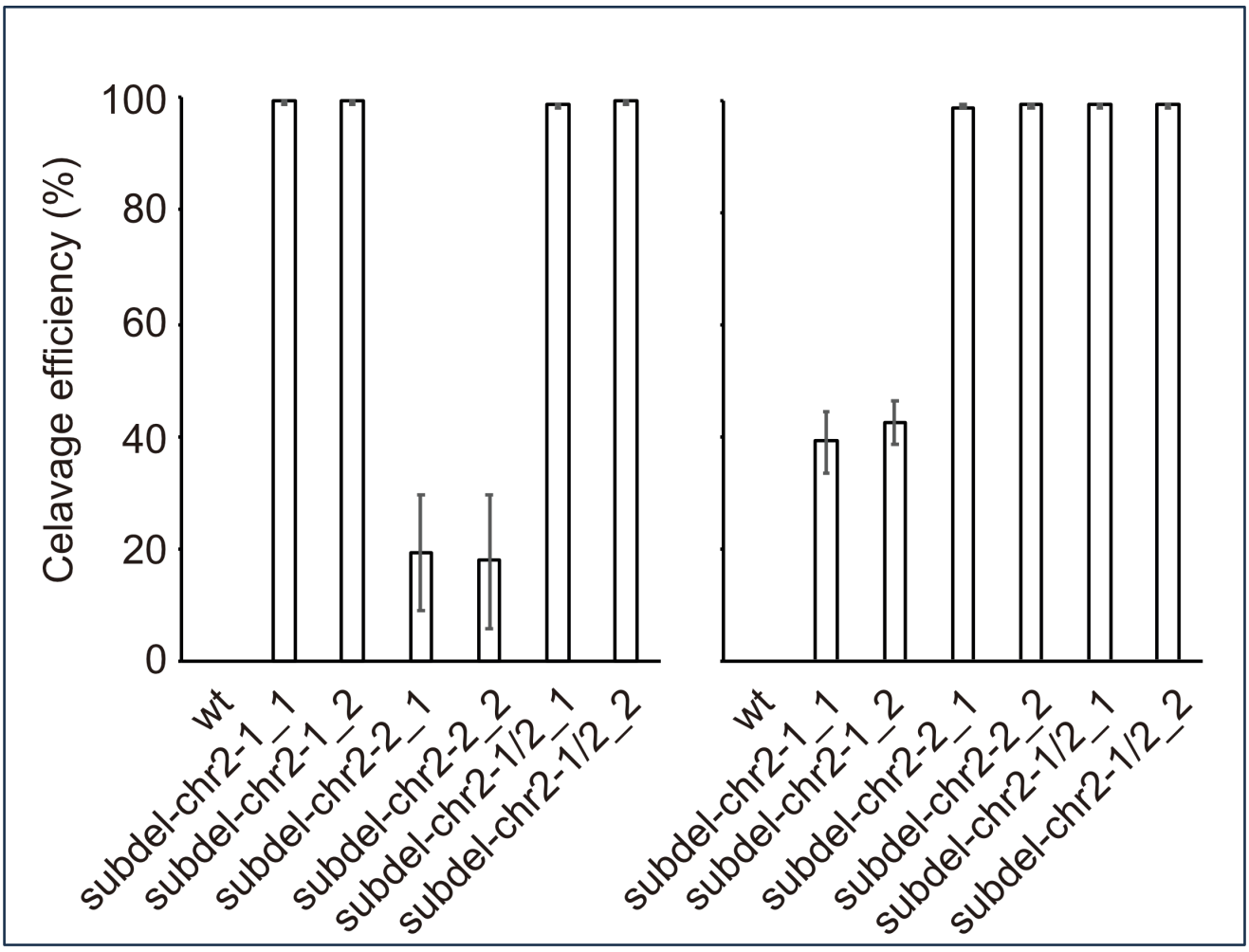

Figure. 3-E: Quantitative PCR. qPCR was conducted to estimate cleavage efficiencies in two biological independent transgenic parasites used in Fig. 1d. The crRNAs used for deleting each subtelomeric region are shown at the top. Data are presented as mean ± SD (n = 3 technical replicates).

The visualized read mapping revealed that no sequencing reads were aligned to the regions downstream of PF3D7_0201300 and PF3D7_0221400 toward the telomeric ends of chromosome 2 in subdel-chr2-1/2, indicating the deletion of subtelomeric regions **(Fig. 3-F)** The sequence analysis of mapping result suggested that the cleavage site was likely on the crRNA designed for PF3D7_0221400, the other was found 13 bp upstream of that for PF3D7_0201300.

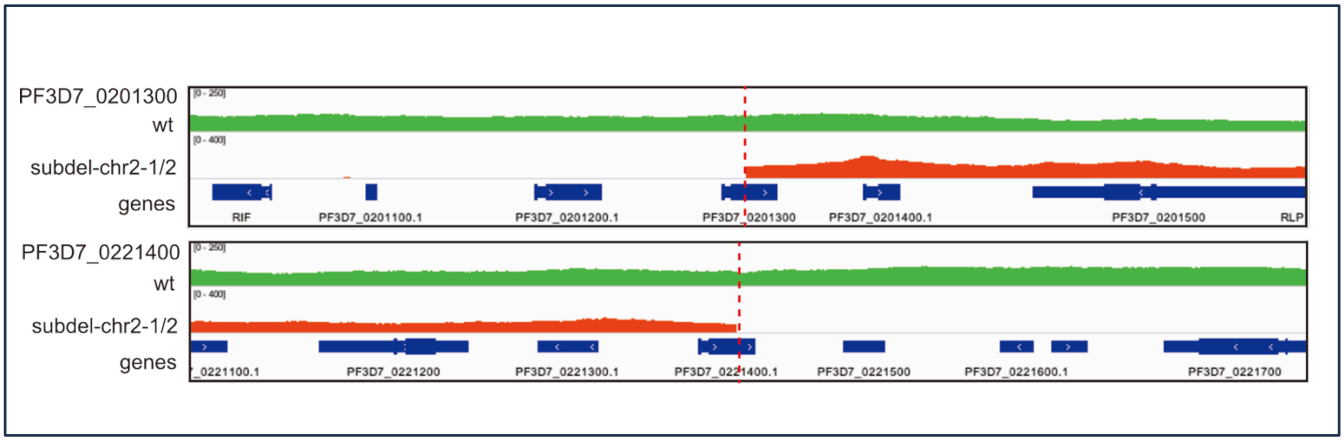

Figure. 3-F: Long-read sequencing mapping of the subtelomeric regions of chromosome 2. Sequencing reads from the wild-type (parental 3D7 strain) and subdel-chr2-1/2 lines are shown in green and red, respectively. Dashed red lines indicate the boundaries of the deleted regions.

To investigate whether *de novo* telomeres were added at the predicted cleavage sites through telomere healing, we constructed a hypothetical genome sequence by adding telomeric repeats at these sites and remapped the obtained long reads to this modified genome **(Fig. 3-G).** The reads were seamlessly mapped to the modified genomic sequences, indicating the addition and extension of telomeric repeats at the predicted cleavage sites. These results suggest that telomere healing occurred following AsCas12-UL-mediated cleavage, as expected.

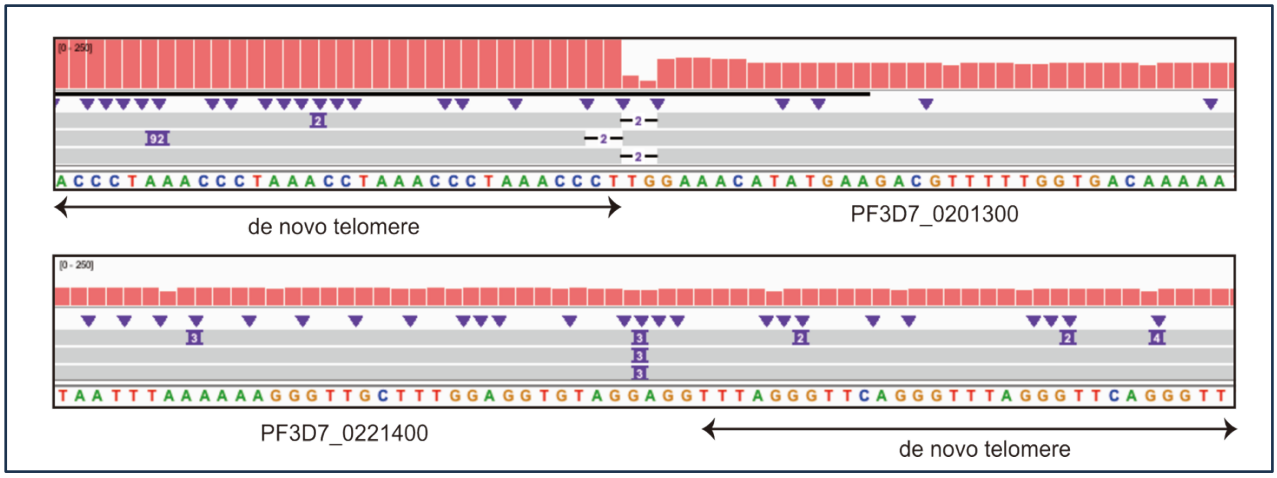

Figure. 3-G: The addition of *de novo* telomeres at the boundaries of the deleted regions is indicated by arrows. Three representative long-read sequencing reads are shown for each chromosome end, mapped across the deletion boundaries, further supporting the occurrence of telomere healing.

3.6 Simultaneous removal of multiple subtelomeric regions

Given that *P. falciparum* possesses 14 haploid genomes, there are a total of 28 subtelomeric regions. The removal of these regions individually is both labor-intensive and time-consuming. To address this, we aimed to simultaneously excise multiple subtelomeric regions using pCas12-UL in combination with tandemly arrayed crRNAs. Seven crRNAs targeting PF3D7_0532900, PF3D7_0937300, PF3D7_1000600, PF3D7_1101300, PF3D7_1149800, PF3D7_1200600, and PF3D7_1479400 were designed using CRISPOR, conjugated with DR spacers in tandem, and subsequently cloned into the pCas12-UL plasmid **(Fig. 3-H).** The resultant plasmid, named pCas12-UL-7crrna, was introduced into *P. falciparum* 3D7 strain, and transgenic parasites with the plasmid were selected by drug screening with pyrimethamine, followed by eliminating the plasmids from them with 5-FC. The obtained transgenic parasite was named subdel-7chr.

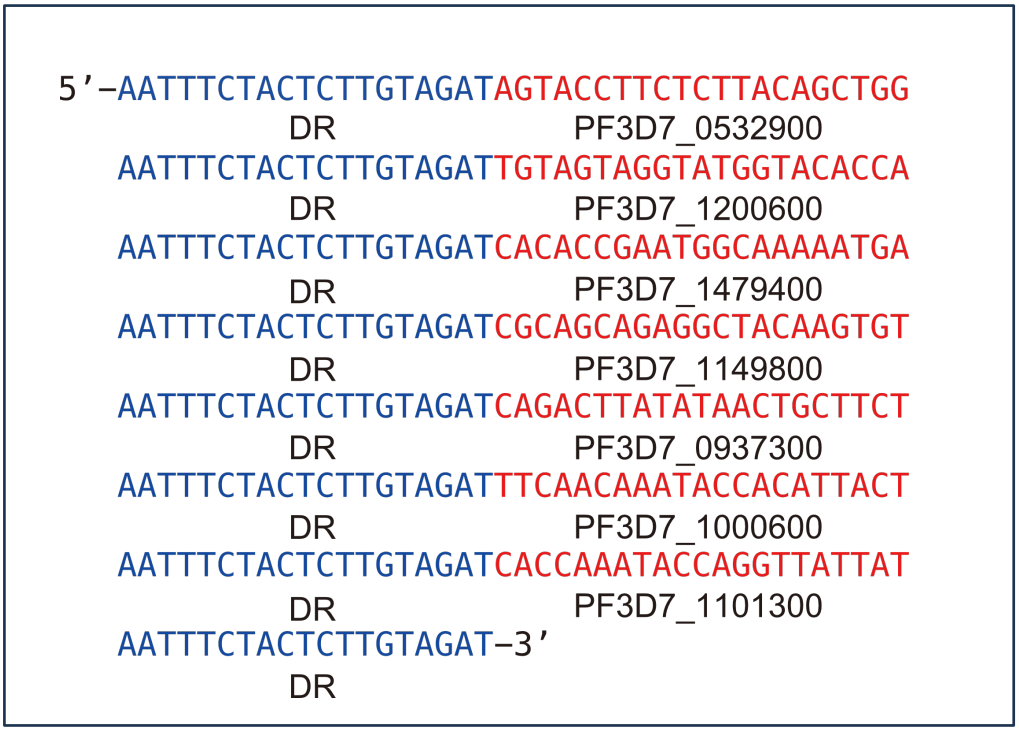

Figure. 3-H: The DNA sequence used to generate seven tandemly arrayed crRNAs is shown. Direct repeats (DR) and individual crRNAs are indicated in blue and red, respectively.

3.7 Confirmation of deletion of multiple subtelomeric regions within subdel-7chr strain

Electrophoresis of genotyping PCR products for each targeted subtelomeric region suggested the cleaving efficiencies were varied among crRNAs **(Fig. 3-I)**.

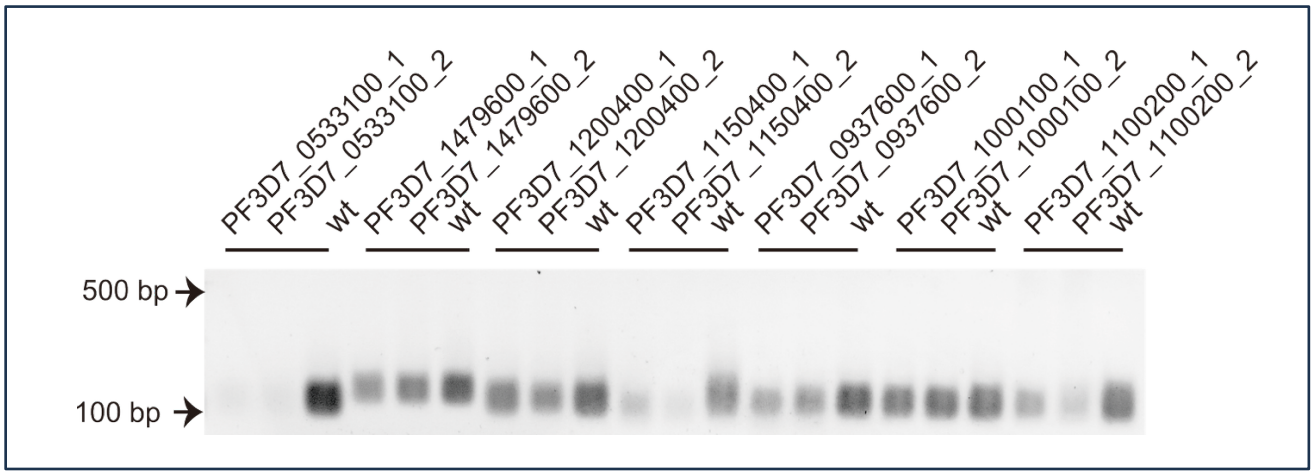

Figure. 3-I: Genotyping PCR was performed for transgenic parasites obtained by two independent transfection experiments. The target genes for each locus are indicated at the top of each well.

Subsequent qPCR analysis for four targeted subtelomeric regions harboring PF3D7_0532900, PF3D7_0937300, PF3D7_1000600, and PF3D7_1479400 located indicated that those cleaving efficiencies at these loci appear to be at least 85 % or higher **(Fig. 3-J)**.

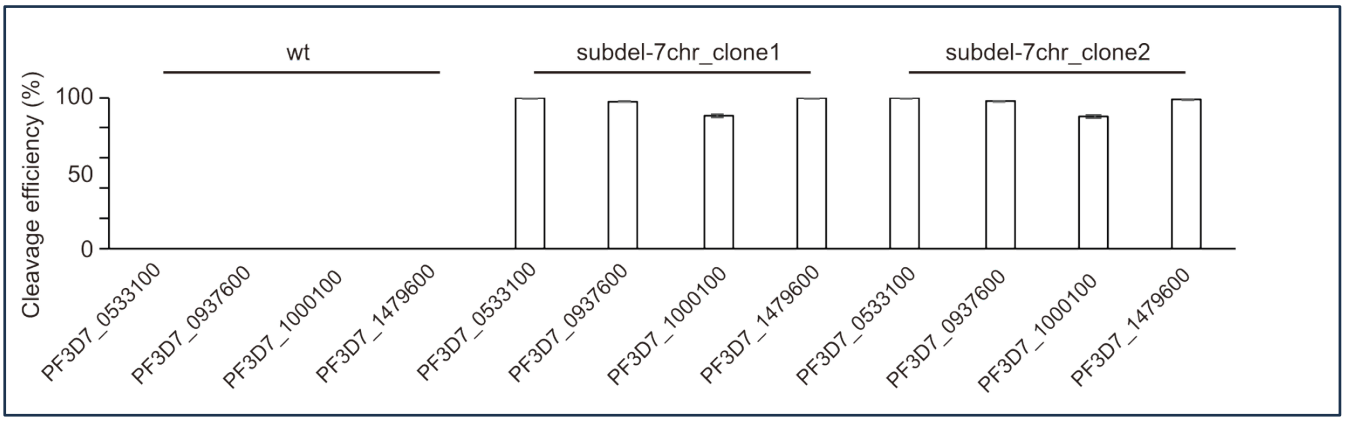

Figure. 3-J: Cleavage efficiencies of four crRNAs targeting chromosomes 5, 9, 10, and 14 were estimated using qPCR from two biologically independent parasite samples used in (Figure. 3-I). The qPCR target loci are indicated at the bottom. Data are presented as mean ± SD (n = 3 technical replicates).

Fourteen clonal parasite lines were derived from two biologically independent transfected populations, and genotyping PCR of them suggested that all seven targeted subtelomeric regions were likely deleted in four of these clones. Long-read sequencing of the representative clone confirmed the deletion of all targeted subtelomeric regions, demonstrating the feasibility of simultaneous removal of multiple subtelomeric regions in a single experiment using pCas12-UL **(Fig. 3-K)**.

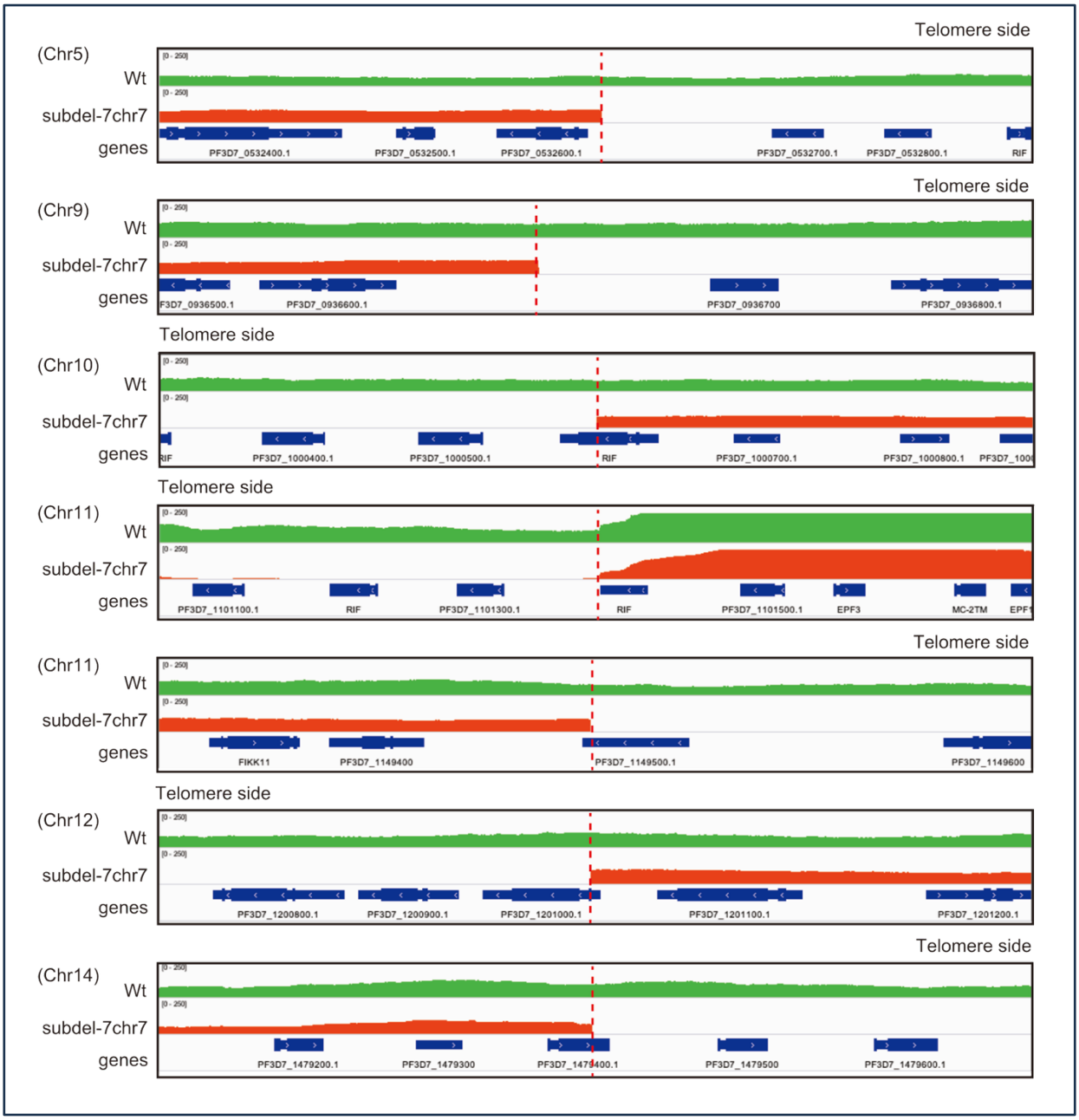

Figure. 3-K: Long-read sequencing results of the subtelomeric regions targeted by the seven tandemly arrayed crRNAs are shown. Sequencing reads from the wild-type (parental 3D7 strain) and subdel-7chr clonal lines are displayed in green and red, respectively. Dashed red lines indicate the boundaries of the deleted regions.

CHAPTER 4 - DISCUSSION

VSAs, which play a critical role in the pathogenesis of severe malaria syndromes, are encoded by large multigene families consisting of dozens to hundreds of genes primarily located in heterochromatic regions such as subtelomeric domains. These genomic loci are notoriously difficult to manipulate genetically due to their structural complexity and high recombinogenic nature. Furthermore, *P. falciparum* exhibits antigenic variation by randomly switching VSA expression through poorly understood epigenetic mechanisms, making it challenging to obtain parasite lines that stably express a specific VSA for experimental purposes(13), (5). Many attempts have been made to study the regulation and function of VSA genes through genetic deletion or disruption. One of the earliest efforts was reported by Tomasino *et al*, who developed a transfection system to induce targeted deletions in the subtelomeric regions of *Plasmodium* chromosomes using linear DNA constructs designed to excise terminal chromosomal sequences(35). Horrocks and colleagues later employed insertional mutagenesis to disrupt individual *var* genes, such as A4var on chromosome 13, providing insights into transcriptional regulation and gene switching behavior(36). And the same research group also documented spontaneous subtelomeric deletions in the *P. falciparum* genome, which led to the loss of specific *var* genes and phenotypic changes, highlighting the intrinsic adaptability parasite(37). More recently, Bryant *et al*. applied CRISPR/Cas9 to delete intronic regions within the *var2csa* gene. Although this strategy demonstrated proof-of-concept for editing var genes, it was also associated with unintended telomeric mutations and interchromosomal recombination events, emphasizing the limitations of Cas9-based editing in subtelomeric regions(38).

The present study is, to my knowledge, the first to apply AsCas12a Ultra for the targeted removal of subtelomeric VSA gene regions in *P. falciparum*, and to establish a reproducible episome-free genome editing protocol. When comparing my data to the study by Zhang *et al* (26), which demonstrated ~100% cleavage efficiency across diverse human cell types using AsCas12a Ultra, my study *P. falciparum* wild-type (WT) constructs exhibit similarly high cleavage efficiency (~100%). In contrast, cleavage efficiency in

sub-deletion constructs significantly dropped to ~25–45%. This reduction likely reflects interference with AsCas12a Ultra activity due to changes in chromatin structure, altered guide RNA targeting sites, or reduced DNA accessibility in the modified subtelomeric context.

These findings underscore the superior efficiency and robustness of AsCas12a Ultra in comparison to earlier CRISPR/Cas9-based methods, especially in genetically challenging regions such as the subtelomeric domains. While previous systems showed variable efficiency and unintended genomic alterations, the AsCas12a Ultra platform consistently achieves high editing rates without detectable off-target effects or chromosomal aberrations, making it an ideal tool for functional genomics studies targeting the var gene family and other VSA-encoding loci.

In conclusion, the approach combining AsCas12a-ultra and telomere healing is useful for removing subtelomeric region containing VSAs and may allow for the complete removal of all subtelomeric regions with minimal experimental effort. Conversely, approximately 37.7% of *var*, 4.5% of *rifin*, and 3.1% of *stevor* genes are positioned in internal chromosomal regions that are characterized by heterochromatin(15). Therefore, to generate VSA-null parasite, those VSA genes must be removed**.** In a previous study, we demonstrated that chromosomes can be split at a specific site by cleavage with SpCas9 nuclease, followed by repair using a DNA fragment containing both a centromere and a telomere(39). Therefore, the use of AsCas12a-ultra in place of SpCas9 may enable the removal of internal chromosomal VSA genes in a manner analogous to the chromosome-splitting technique.

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ACKNOWLEDGEMENTS

In accordance with the Islam traditions, I express my gratitude toward almighty Allah for giving me the power to finish this work. I would also express my respect and deep gratitude to my research supervisors, Prof. Tetsuya Iida and Prof. Shiroh Iwanaga for their kind support and great guidance through the completion of this PhD thesis.

For sure, I am very grateful for my dear parents, who praised and encouraged me all the time.

All thanks to the sponsor of my PhD project, the (Monbukagakusho scholarship) of the Ministry of Education, Culture, Sports, Science (MEXT), which generously funded and supported this project.

This work also was funded by Japan Science and Technology (S.I. CREST, JPMJCR23B5) and Japan Society for the Promotion of Science (S.I. 24K22055).

AUTHOR ACHIEVMENTS

List of publications

* Amin A. Mohamed Musa, Sondos A. Abd Alrahem , **Shymaa A. Saeed** , AltayebAbdulmonem , Khalid A. Mohamed , Mamoun M A Homeida. Prevalence and Associations of Hepatitis B Virus Infection among Students of Traditional Schools, East Nile Locality,Khartoum, Sudan, American Journal of Microbiology and Immunology (AJMI), 6:10- September 2021. https://escipub.com/ajmi-2021-08-0805/

* **Shymaa A. Saeed**, Farahana Kresno Dewayanti , Yuki Nishimura, Tetsuya Iida, and Shiroh Iwanaga. Method for deletion of variant surface antigen genes at subtelomeric region of *Plasmodium falciparum*. (Manuscript is submitted to Parasitology International journal)

Conferences (oral and poster presentations)

* **Shymaa Ali**, Shimpei Gotoh, Takashi Nishida, and Yasuhiko Horiguchi, Identification of virulence factors required for colonization of Bordetella during infection. 16th Wakate Colosseum for Bacteriology, Sapporo, Hokkaido, Japan, Aug 2022 (Oral Presentation)

***Shymaa Ali**, Shimpei Gotoh, Takashi Nishida, and Yasuhiko Horiguchi, Identification of virulence factors required for colonization of Bordetella during infection. 75th Kansai meeting of Japanese Society of Bacteriology, Kyoto University, Kyoto, Japan, Nov 2022 (Oral Presentation).

***Shymaa Ali**, Shimpei Gotoh, Takashi Nishida, and Yasuhiko Horiguchi, Identification of virulence factors required for colonization of Bordetella during infection. 96th Meeting of Japanese Society of Bacteriology, Himeji, Hyogo, Japan, 2023 (poster Presentation)

Academic Awards

Won six academic prizes during the five years of studying at the Faculty of Medical Laboratory Sciences (FMLS)- University of Khartoum- Khartoum city, Sudan classified as follows:

-Awarded academic prize for the best academic performance (top student)

for 5 successive years

1. First-year prize (2011-2012)
2. Second-year prize (2012-2013)
3. Third-year prize (2013-2014)
4. Fourth-year prize (2014-2015)
5. Awarded the university prize for best academic performance (top student) in the Department of (Medical Microbiology and Immunology) in the final (fifth year / 2015-2016)
6. Awarded the college prize for the second-best academic performance at the level of all Departments (Medical Microbiology and Immunology, Hematology, Parasitology and Medical Entomology, Histopathology, and Clinical Chemistry) for the fifth level (2015-2016).