

Identification and Functional Characterization of a Novel Component of the DNA Repair Machinery of Malaria Parasites

A thesis submitted during 2025 to the University of Hyderabad in partial fulfilment of the award of a Ph.D. degree in the

Department of Biochemistry

School of Life Sciences

By

**Abhilasha Gahlawat
(19LBPH01)**



**Department of Biochemistry
School of Life Sciences
University of Hyderabad
(P.O) Central University,
Gachibowli, Hyderabad, 500046,
Telangana, INDIA**

Oct 2025



University of Hyderabad
(Central University established in 1974 by Act of Parliament)
School of Life Sciences
Department of Biochemistry
Hyderabad-500046, INDIA

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This is to certify that this thesis entitled “**Identification and Functional Characterization of a Novel Component of the DNA Repair Machinery of Malaria Parasites**” submitted by **Abhilasha Gahlawat**, bearing Registration number 19LBPH01 in partial fulfilment of the requirements for award of Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences, is bonafide work carried out by him under my guidance and supervision.

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Further, the student has passed the following courses towards the fulfilment of the coursework requirement for Ph.D.

Course Code	Name	Credits	Pass/Fail
SL 801	Research Methodology/Analytical Techniques	4	Pass
SL 802	Research Ethics, Biosafety, Biostatistics and Data Analysis	4	Pass
SL 803	Research Proposal and Scientific Writing	4	Pass
SL 804	Research and Publication Ethics	4	Pass



(Supervisor)



(Head of the Department)



(Dean of the School)

Dr. MRINAL KANTI BHATTACHARYYA PhD
PROFESSOR
DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046. INDIA

अध्यक्ष / Head
जीवरसायनविभाग / Department of Biochemistry
जीवविज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500046. INDIA

संकाय अध्यक्ष / DEAN
जीव विज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500046.



University of Hyderabad
(Central University established in 1974 by Act of Parliament)
School of Life Sciences
Department of Biochemistry
Hyderabad-500046, INDIA

DECLARATION

I, **Abhilasha Gahlawat**, hereby declare that this thesis entitled **“Identification and Functional Characterization of a Novel Component of the DNA Repair Machinery of Malaria Parasites”** submitted by me under the guidance and supervision of **Prof. Mrinal Kanti Bhattacharyya** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. I hereby agree that my thesis can be deposited in Shodhganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed.

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(Prof. Mrinal Kanti Bhattacharyya)

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(19LBPH01)

Dr. MRINAL KANTI BHATTACHARYYA PhD
PROFESSOR
DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046, INDIA

SDG (SUSTAINABLE DEVELOPEMENT GOALS)

Thesis title: Identification and Functional Characterization of a Novel Component of the DNA Repair Machinery of Malaria Parasites

Student Regd. No. : 19LBPH01

Supervisor : Prof. Mrinal Kanti Bhattacharyya

Department : Department of Biochemistry

School : School of Life Sciences

This Ph.D. research project, "*Identification and Functional Characterization of a Novel Component of the DNA Repair Machinery of Malaria Parasites*", is closely aligned with the United Nations Sustainable Development Goals (SDGs). Malaria, a Neglected Tropical Disease (NTD), continues to impose a significant health and economic burden, particularly in endemic regions.

The study primarily supports **SDG 3 (Good Health and Well-Being)** by advancing knowledge of DNA repair mechanisms in *Plasmodium falciparum*. By identifying a parasite-specific DNA repair protein with potential as a novel therapeutic target, this work contributes directly to the sub-goal **SDG 3.3**, which calls for ending the epidemics of AIDS, tuberculosis, malaria, and other neglected tropical diseases by 2030. Such findings hold promise for the development of innovative anti-malarial strategies aimed at overcoming current challenges such as drug resistance.

Beyond health outcomes, the project also addresses **SDG 9 (Industry, Innovation, and Infrastructure)** through its emphasis on translational research and biotechnology innovation by discovering novel targetable *Plasmodium*-specific proteins to combat malaria. By creating knowledge that can drive therapeutic discovery, this work supports sub-goal **SDG 9.5**, which highlights strengthening scientific research, enhancing technological capacity, and fostering innovation in developing countries.

Additionally, the translational impact of this study aligns with **SDG 8 (Decent Work and Economic Growth)**, particularly the sub-goal **SDG 8.2**, by promoting innovation and laying the foundation for biotechnology-driven solutions that enhance economic productivity and support long-term growth. In summary, this research advances both global health and innovation agendas, linking molecular parasitology to sustainable development and the UN 2030 vision.



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Department of Biochemistry
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Supervisor

(Prof. Mrinal Kanti Bhattacharyya)

Dr. MRINAL KANTI BHATTACHARYYA PhD
PROFESSOR
DEPARTMENT OF BIOCHEMISTRY
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046, INDIA

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Abbreviations

Ade: Adenine

Amp: Amp

cDNA: Complementary DNA

Da: Dalton

DEPC: Diethyl pyrocarbonate

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

DSB: Double strand break

DTT: Dithiothreitol

EDTA: Ethylene diamine tetra acetic aci

HR: Homologous Recombination

kDa: Kilo Dalton

LB: Luria-bertani broth

Leu: Leucine

LiOAc: Lithium Acetate

M: Molar

mg: Milli gram

mM: Milli molar

MMS: Methyl Methane Sulfonate

mL: Milli liter

NaCl: Sodium Chloride

NaOAc: Sodium Acetate

NaOH: Sodium Hydroxide

ng: nano gram

OD: Optical Density

ORF: Open Reading Frame

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PMSF: Phenyl-methane-sulphonyl-fluoride

PVDF: Poly vinylidene Flouride

q-PCR: Quantitative PCR

RBC: Red Blood Cell

RNA: Ribonucleic acid

RNase: Ribonuclease

rpm: rotation per minute

RPMI: Roswell park memorial institute medium

RT: Room temperature

RT-PCR: Reverse Transcriptase – Polymerase Chain Reaction

SC: Synthetic Complete

SDS: Sodium Dodecyl sulfate

SDS-PAGE: Sodium Dodecyl sulfate Polyacrylamide gel electrophoresis

ssDNA: Single strand DNA (Salmon sperm DNA)

TBE: Tris Borate EDTA

TCA: Trichloroacetic acid

TE: Tris EDTA

TRP: Tryptophan

Ura: Uracil

µg: Microgram

μL: Microliter

WHO: World Health Organization

YPD: Yeast Peptone Dextrose

Chapter 1

Introduction

1.1 Malaria Overview.

Malaria is a serious and potentially life-threatening disease. It is one of the deadliest vector-borne infectious diseases in human history, caused by protozoan parasites of the genus *Plasmodium* and transmitted via the bite of infected female *Anopheles* mosquitoes. Its devastating impact spans centuries, shaping human populations and influencing historical events. Its name comes from the Italian word *mal aria* (“bad air”), reflecting the old belief that swamp vapours caused the illness. The disease was first linked to parasites in human blood in 1880, when French military physician Charles Louis Alphonse Laveran discovered *Plasmodium* in the blood of malaria patients—an achievement that earned him the 1907 Nobel Prize in Medicine (1). Later, in 1897, Sir Ronald Ross demonstrated the role of *Anopheles* mosquitoes in transmission, laying the foundation for vector control strategies and receiving the Nobel Prize in 1902 (2). This understanding paved the way for modern prevention and control efforts. However, it still remains one of the most significant global public health concerns and the disease continues to impose substantial morbidity, mortality, and socio-economic burden worldwide (3).

1.1.1 World Malaria Burden.

Malaria remains a substantial global health concern, with its burden concentrated in tropical and subtropical regions (3). According to the 2024 World Malaria Report by the World Health Organization (WHO), the disease was responsible for an estimated 263 million cases and over 597,000 deaths in 2023 alone (3). Despite notable progress over the past two decades—largely attributed to the deployment of artemisinin-based combination therapies (ACTs) and large-scale vector control interventions—recent trends indicate a stagnation in progress (4). This plateau is largely driven by the emergence and spread of antimalarial drug resistance, increasing insecticide resistance, and various socioeconomic disruptions that compromise intervention efficacy (5). The parasites’ high genetic adaptability allows them to develop drug resistance and alter host specificity, posing challenges for eradication efforts (6,7). These evolving

challenges underscore the pressing need for novel malaria interventions, including the identification of new therapeutic targets to guide next-generation antimalarial drug development.

1.1.2 Causative Agent.

Malaria is caused by protozoan parasites of the genus *Plasmodium*, transmitted to humans through the bite of infected *Anopheles* mosquitoes. Over 200 *Plasmodium* species have been identified, but only five *Plasmodium* species are known to infect humans naturally, namely: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (subspecies *P. ovale curtisi* and *P. ovale wallikeri*), and *P. knowlesi* (8). Among these, *P. falciparum* is the most virulent and is responsible for the majority of severe malaria cases and deaths globally and *P. vivax* is more geographically widespread but typically less deadly. The parasite has a complex life cycle involving both the human host and the mosquito vector, including asexual replication in human hepatocytes and erythrocytes, and sexual reproduction in the mosquito midgut (9).

1.1.3 Vector.

Malaria is transmitted exclusively by the bite of infected female *Anopheles* mosquitoes. Over 450 species of *Anopheles* have been identified, but only about 40 are considered significant vectors for human malaria transmission (10). Major vector species vary geographically—for instance, *Anopheles gambiae* and *An. funestus* dominate in sub-Saharan Africa, while *An. stephensi* and *An. culicifacies* are important in South Asia (11). The mosquito acquires *Plasmodium* gametocytes during a blood meal, where they undergo sexual reproduction and develop into sporozoites that migrate to the salivary glands, ready for transmission. These mosquitoes are most active during evening and night-time hours, favour warm, humid environments, and breed in freshwater habitats. Their efficiency as vectors is influenced by factors such as feeding behaviour, longevity, and susceptibility to *Plasmodium* infection. Control strategies, such as insecticide-treated nets and indoor residual spraying, target these behaviours to reduce transmission.

1.1.4 Life Cycle of *Plasmodium falciparum*.

The life cycle of *Plasmodium falciparum* spans two hosts—humans and *Anopheles* mosquitoes—and alternates between sexual and asexual reproduction (Figure 1.1). Infection begins when an infected female *Anopheles* mosquito injects sporozoites into the human bloodstream during a blood meal. These sporozoites rapidly migrate to the liver and invade hepatocytes, where they undergo exoerythrocytic schizogony over 7–10 days. Each liver-stage schizont produces thousands of merozoites, which are released into the bloodstream upon rupture of the hepatocytes.

In the erythrocytic cycle, merozoites invade red blood cells (RBCs) and develop through three distinct stages:

1. Ring stage (early trophozoite) – a small ring-shaped form visible in RBCs, marking the beginning of parasite metabolism. This represents the G1 phase of the cell cycle and does not
2. Trophozoite stage – the parasite grows in size, actively metabolizing haemoglobin, and the host cell enlarges.
3. Schizont stage – the mature form containing multiple merozoites; upon rupture, these merozoites are released to infect new RBCs, perpetuating the asexual cycle and causing the periodic fevers characteristic of malaria.

A subset of merozoites differentiate into male (microgametocytes) and female (macrogametocytes) gametocytes, which circulate in the human blood. When another mosquito feeds on an infected person, it ingests these gametocytes. In the mosquito midgut, gametocytes undergo sexual reproduction to form a zygote, which develops into a motile ookinete. The ookinete penetrates the gut wall and forms an oocyst, where sporogony occurs to produce

thousands of sporozoites. These sporozoites migrate to the mosquito's salivary glands, ready to infect a new human host, thus completing the cycle (12,13).

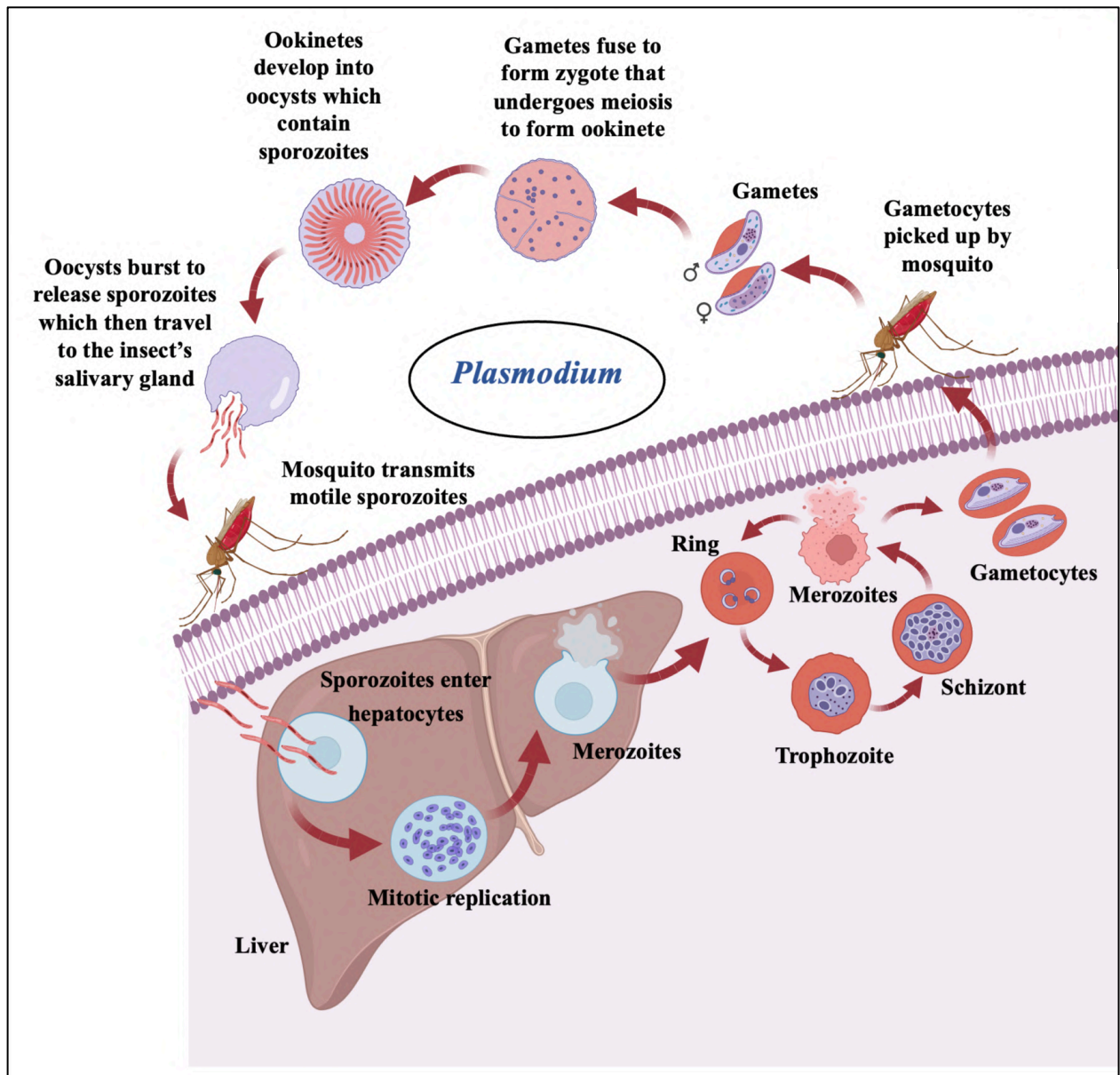


Figure 1.1: Illustration representing the complete life cycle of *Plasmodium falciparum*.

1.1.5 Human Host and Pathogenesis.

Humans serve as the definitive vertebrate host for *Plasmodium*. Infection begins when an infected mosquito injects sporozoites into the skin during a blood meal. These sporozoites travel via the bloodstream to the liver, invade hepatocytes, and undergo schizogony to produce merozoites. Merozoites are released into the bloodstream, invading red blood cells and initiating the erythrocytic cycle, which is responsible for the clinical manifestations of the disease (14).

The severity of malaria largely depends on the *Plasmodium* species and host factors, including immunity, age, and comorbidities. *P. falciparum* is particularly pathogenic due to its ability to cause sequestration of infected erythrocytes in microvasculature, leading to cerebral malaria, severe anaemia, acidosis, and multi-organ failure.

1.1.6 Clinical features of Malaria.

The clinical manifestations of malaria arise primarily from the asexual erythrocytic stage of the *Plasmodium* life cycle, during which merozoites invade red blood cells (RBCs), replicate, and rupture them, releasing new merozoites and parasite by-products into circulation. This cyclical process triggers characteristic clinical symptoms, ranging from mild febrile illness to severe, life-threatening complications depending on the *Plasmodium* species, host immunity, and co-morbid conditions. The classical symptoms of malaria include fever, chills, sweating, headache, malaise, muscle and joint pain, nausea and vomiting, diarrhea (sometimes), splenomegaly (enlarged spleen) and hepatomegaly (enlarged liver, less common). The incubation period typically ranges from 7 to 30 days, depending on the *Plasmodium* species (15). Severe malaria (mostly presented by *P. falciparum*) can present with complications such as:

1. Cerebral malaria (seizures, altered consciousness, coma)
2. Severe anaemia (due to haemolysis of infected and uninfected erythrocytes)
3. Acute kidney injury
4. Respiratory distress or pulmonary edema
5. Metabolic acidosis
6. Jaundice
7. Hypoglycaemia
8. Shock and multi-organ failure

Relapses can occur in *P. vivax* and *P. ovale* infections due to dormant hypnozoites in the liver.

1.2 Current Antimalarials and the Need for Novel Drug Targets.

1.2.1 Current Antimalarial Therapies.

The treatment of malaria has evolved significantly over the past century, with several classes of antimalarial drugs targeting different stages of the *Plasmodium* life cycle. Currently, the World Health Organization (WHO) recommends artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated *Plasmodium falciparum* malaria, owing to their rapid parasite clearance and reduced likelihood of resistance when used in combination (11). Key antimalarial drug classes include:

1. Artemisinin derivatives (artemether, artesunate, dihydroartemisinin): Potent schizonticidal drugs with rapid action, typically combined with a partner drug such as lumefantrine, piperaquine, or amodiaquine.
2. Quinolines (chloroquine, quinine, mefloquine, amodiaquine): Traditionally effective against blood-stage parasites, though widespread chloroquine resistance has limited its use against *P. falciparum*.
3. Antifolates (sulfadoxine-pyrimethamine): Inhibit folate biosynthesis in parasites, used in intermittent preventive treatment, though resistance is common.
4. Atovaquone-proguanil: Targets the parasite mitochondrial electron transport chain and folate pathway, often used for prophylaxis in travellers.
5. Primaquine and tafenoquine: Effective against dormant hypnozoite stages of *P. vivax* and *P. ovale*, preventing relapses.

Despite these advances, resistance has emerged to nearly every antimalarial drug introduced, posing a significant threat to malaria control and eradication programs.

1.2.2 Emergence of Drug Resistance.

Drug resistance in *P. falciparum* has been documented against chloroquine, sulfadoxine-pyrimethamine, mefloquine, and more recently artemisinin derivatives (16,17). Artemisinin resistance, first detected in the Greater Mekong Subregion, is characterized by delayed parasite clearance, often linked to mutations in the *kelch13* gene (18). Table 1.1 highlights the current status of resistance against each mentioned commercial antimalarial and the respective genetic marker responsible for the acquired resistance. Resistance to partner drugs, such as piperaquine and lumefantrine, further compromises ACT efficacy. The spread of multidrug-resistant *P. falciparum* strains threatens to reverse decades of progress in malaria reduction. Similarly, *P. vivax* has shown increasing chloroquine resistance in several endemic regions (19). This growing resistance underscores the fragility of current therapeutic options.

Drug Name	Status of resistance	Genetic markers for resistance
Chloroquine, Quinine, Mefloquine, Halofantrine	Yes	Point mutation in <i>Pfcr1</i> , <i>Pfmdr1</i> , <i>Pfmrp</i>
Sulfadoxine, Sulfene	Yes	Point mutation in <i>Pfdhps</i>
Pyrimethamine, Proguanil	Yes	Point mutation in <i>Pfdhfr</i>
Artemisinin	Yes	Polymorphism in Kelch 13 protein
Primaquine	Yes	Not known
Atovaquone	Yes	Point mutation in <i>cytb</i> gene

Table 1.1: List of common anti-malarial drugs, their status of resistance and genetic markers responsible for the resistance in each case.

1.2.3 Antigenic Variation and Immune Evasion.

A major obstacle in malaria control is the parasite's ability to undergo antigenic variation, allowing it to evade host immune responses. In *P. falciparum*, this is mediated by the *var* gene family, encoding the surface antigen PfEMP1, which undergoes clonal variation and facilitates cytoadherence to host endothelial cells (20). Antigenic variation complicates vaccine development and contributes to recurrent infections, as host immunity is strain-specific and often short-lived. This dynamic antigenic landscape makes it difficult for existing immunological and chemotherapeutic strategies to achieve long-term efficacy, further reinforcing the need for novel therapeutic approaches.

1.2.4 The Need for Novel Drug Targets.

Although artemisinin-based combination therapies (ACTs) have significantly contributed to lowering malaria-related illness and death, the persistent rise of drug-resistant strains and the parasite's complex immune evasion strategies driven by antigenic variation continue to undermine these achievements. Although recent milestones include the development of malaria vaccines—RTS,S/AS01 (Mosquirix) and R21/Matrix-M which represent landmark achievements in parasite immunology, their protective efficacy remains moderate and requires integration with existing control measures (11). Most importantly, these current vaccines are only effective for infants and children, and this calls for future malaria control efforts that must prioritize the discovery and clinical validation of novel drug targets that can act on essential and less mutable parasite pathways, combined with innovative strategies for resistance surveillance and integrated control programs.

1.2.5 *Plasmodium* genome and DNA double strand breaks.

Plenty of DNA double-strand breaks (DSBs) occur naturally within the genome of malaria parasites during its asexual stages in the human host due to a number of reasons (21,22). The *Plasmodium* genome is highly vulnerable to DNA damage due to its high AT content and numerous DNA DSBs are generated within its genome due to errors during replication like replication fork arrest or due to the generation of free radicals produced during pathways like haemoglobin detoxification or artemisinin activation (21–23). In *Plasmodium* parasites, haemoglobin digestion within the food vacuole releases free heme (ferriprotoporphyrin IX), which catalyses the formation of reactive oxygen species (ROS), leading to oxidative stress (24). The parasite detoxifies this by polymerizing heme into inert hemozoin, but some free heme and ROS still persist and contribute to cellular damage. In another pathway, in presence of artemisinin, this antimalarial gets activated in *Plasmodium* parasites by interacting with free or loosely bound ferrous iron (Fe^{2+}) from digested haemoglobin (25). This activation cleaves

artemisinin's endoperoxide bridge, generating reactive oxygen species (ROS) and carbon-centered free radicals that damage parasite proteins, membranes, and organelles. Additionally, reactive oxygen species and free radicals can cause DNA double-strand breaks (DSBs) primarily by attacking the sugar-phosphate backbone and nucleotide bases of DNA. Hydroxyl radicals, in particular, are highly reactive and can abstract hydrogen atoms from deoxyribose sugars, leading to single-strand breaks that, when closely spaced on opposite strands, result in DSBs (26). Additionally, oxidative modifications of bases like guanine can destabilize DNA structure and promote strand cleavage(27). This creates an inevitable pressure on malaria parasites to evolve coping mechanisms that are able to repair a plethora of DNA DSBs experienced during their asexual growth in order to survive in the human host.

1.3 DNA DSB Repair Mechanism adopted by *P. falciparum*.

As mentioned earlier, *P. falciparum* faces frequent DNA damage during its complex life cycle, including highly lethal DNA DSBs. Remarkably, a single unrepaired DNA double-strand break can prove fatal to a unicellular organism (28). The two mechanisms that operate in eukaryotic organisms to repair these DNA DSBs include Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) (29). NHEJ is completely absent in *Plasmodium* (30,31) making homologous recombination the only prominent indispensable DNA Repair machinery adopted by *Plasmodium* (23) to repair its DNA double-strand breaks. *P. falciparum* lacks the classical non-homologous end joining (C-NHEJ) pathway because its genome does not encode the core components required for this repair mechanism, such as Ku70/Ku80, DNA-PKcs, and XRCC4-Ligase IV (22). These proteins are essential in higher eukaryotes for recognizing DNA double-strand breaks (DSBs) and directly ligating the broken ends. Genomic analysis of *P. falciparum* reveal the presence of homologous recombination (HR)-related proteins like Rad51, Rad54, and Mre11, but none of the canonical NHEJ factors, indicating evolutionary loss of this pathway (31). Instead, the parasite primarily relies on HR for accurate DSB repair and to some

extent on alternative end joining (A-EJ)/microhomology-mediated end joining (MMEJ) when homologous templates are unavailable. Homologous recombination (HR) is a high-fidelity DNA DSB repair mechanism that resolves double-strand breaks by using a homologous DNA sequence—typically the sister chromatid—as a template for accurate repair.

1.3.1 Homologous Recombination pathway in higher eukaryotes.

Homologous recombination is a high-fidelity repair pathway responsible for the accurate repair of DNA DSBs, one of the most lethal forms of DNA damage. Unlike error-prone mechanisms such as non-homologous end joining (NHEJ), HR relies on a homologous DNA sequence, typically the sister chromatid, as a template to restore genetic information with minimal sequence alterations (32).

The HR pathway is initiated by sensing and processing of DSBs. The MRE11–RAD50–NBS1 (MRN) complex recognizes the break site and promotes nucleolytic end resection to generate 3' single-stranded DNA (ssDNA) overhangs (33). This ssDNA is immediately coated by replication protein A (RPA), which stabilizes the overhangs and prevents secondary structure formation. Subsequently, the recombinase RAD51 is recruited to ssDNA with the help of mediators such as BRCA2, PALB2, and Rad52, displacing RPA and forming a nucleoprotein filament (34).

This RAD51-ssDNA filament performs a homology search within the sister chromatid and invades the homologous duplex DNA, generating a displacement loop (D-loop). DNA polymerases then extend the invading strand, using the intact chromatid as a template. The second end of the DSB may also anneal to the extended strand, forming a double Holliday junction (dHJ) intermediate. Resolution of Holliday junctions is carried out by specialized endonucleases such as GEN1, SLX1-SLX4, and MUS81-EME1, or through dissolution mediated by the BLM helicase–TOPIII α –RMI1/2 (BTR) complex, which prevents crossovers

and ensures genome stability (35,36). Figure 1.2 represents all conserved key HR effector proteins (or effectors) that function in the HR-mediated DNA DSB repair.

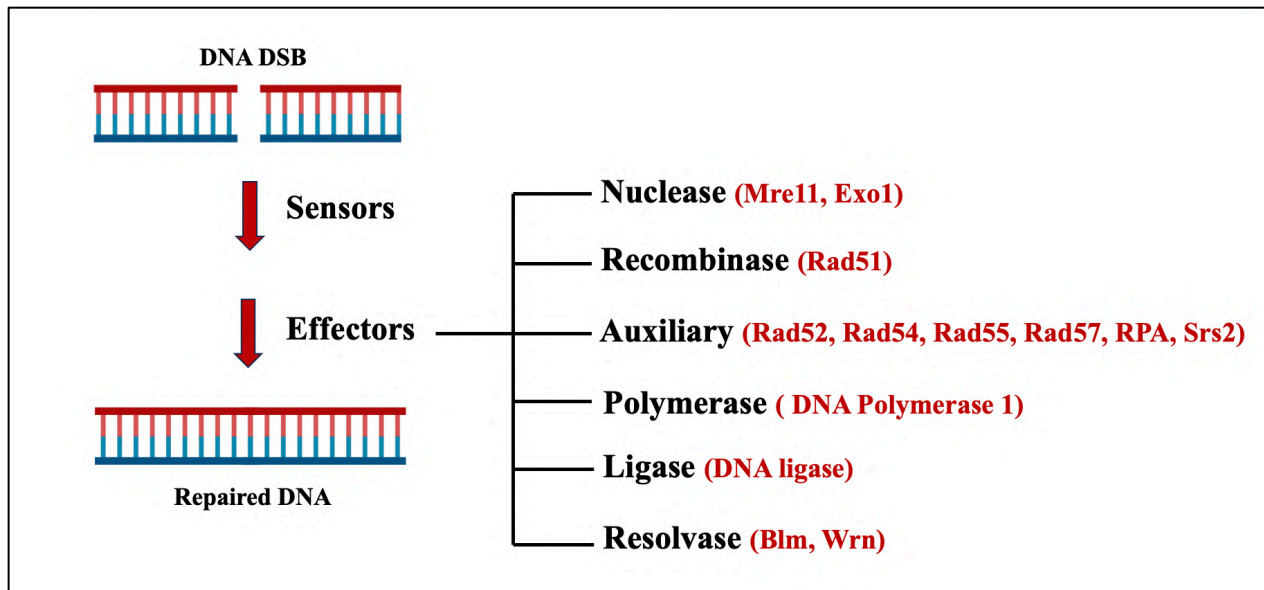


Figure 1.2: Illustration depicting the list of all effector proteins involved in homologous recombination-mediated DNA repair.

Beyond DSB repair, HR is also critical for replication fork restart, meiotic recombination, and telomere maintenance. In meiosis, DMC1, a meiosis-specific recombinase, works in conjunction with RAD51 to promote crossover recombination, ensuring proper chromosome segregation (37). Regulation of HR is tightly controlled: for instance, p53, BLM, and the Fanconi anemia pathway proteins modulate HR activity to balance repair accuracy with genome stability. Defects in HR proteins, including BRCA1/2, RAD51 paralogs, and BLM, lead to genomic instability syndromes and predisposition to cancer, highlighting its essential role in maintaining genome integrity (38)

1.3.2 Homologous Recombination proteins of *P. falciparum*.

The homologous recombination mediated DNA repair proteins in *P. falciparum* have been previously described and implicated as a prospective druggable targets in managing malaria (23,30,39,40). The MRE11 protein in *P. falciparum* (PfalMRE11) is a conserved nuclease and a core component of the MRN (MRE11–RAD50–NBS1) complex, playing a pivotal role in the recognition and repair of DNA double-strand breaks (DSBs) through homologous recombination (39). The PfalMRE11 as the parasite ortholog, shares ~22% sequence identity with human MRE11, yet retaining a structurally conserved nuclease domain resembling that of yeast Mre11 (39). Functional complementation assays demonstrated that the PfalMRE11 nuclease domain could rescue yeast *mre11* mutants, whereas a D398N point mutation abolished this function, underscoring its essential catalytic role (39). PfalMRE11 was seen to be highly expressed during the mitotically active stages of the parasite (trophozoite and schizont), with its expression further induced upon DNA damage, consistent with its role in the parasite's DNA damage response (39). Interaction studies revealed that PfalMRE11 physically associates with PfRad50 and can also interact with yeast Rad50 and Xrs2, highlighting evolutionary conservation of the MRN complex. Functionally, PfalMRE11 mediates DNA end resection at DSBs, thereby enabling RAD51 filament formation and error-free repair, while also contributing to genome plasticity through recombination in sub-telomeric regions housing virulence gene families such as *var*, thus supporting antigenic variation and immune evasion. PfalMRE11 was studied to be a promising antimalarial drug target, where its inhibition could impair both parasite survival under genotoxic stress and recombination-driven antigenic diversity.

Rad51, the homologous recombination recombinase in *P. falciparum* (PfRad51), is a central player in the repair of DNA double-strand breaks (DSBs), which are as mentioned earlier, frequent during replication and oxidative stress. PfRad51 catalyses ATP-dependent strand

exchange and is upregulated in response to DNA damage, indicating its active role in maintaining genome stability (41). Mechanistically, PfRad51 interacts with cofactors like Rad54 and replication protein A (PfRPA) (42). To dissect the essentiality of Rad51-mediated HR in malaria parasites, a previous research engineered a dominant-negative mutant of *Plasmodium* Rad51 (PfRad51^{K143R}), in which a lysine-to-arginine substitution at position 143 disrupted ATPase activity critical for filament dynamics (30). The mutant protein was shown to incorporate into Rad51 nucleoprotein filaments and inhibit wild-type Rad51 function, leading to defective DSB repair. Expression of PfRad51^{K143R} in *Plasmodium berghei* parasites resulted in increased sensitivity to DNA damaging agents such as methyl methane sulfonate (MMS), accompanied by a marked reduction in HR efficiency (30). Strikingly, parasites expressing the dominant-negative allele exhibited significantly reduced parasite loads and prolonged host survival in murine infection models, highlighting the *in vivo* importance of Rad51-dependent HR for parasite proliferation and virulence (30). Complementary experiments in yeast demonstrated that the mutant protein compromised gene targeting and recombination efficiency, underscoring its potent inhibitory effect on HR(30).

In a compelling study, a small-molecule inhibitor of PfRad51, B02, was identified as a novel antimalarial agent (23). Through *in silico* docking, B02 demonstrated high affinity for PfRad51, effectively inhibiting its ATPase and strand-exchange activities, and preventing formation of PfRad51 foci at DNA damage sites (23). B02 was shown to impair DNA double-strand break repair, leading to accumulation of unrepaired lesions and parasite death. Remarkably, B02 exhibited greater potency against both the drug-sensitive (3D7) strain and the multidrug-resistant (Dd2) strain of *P. falciparum* than against mammalian cells, highlighting its therapeutic selectivity (23). Furthermore, B02 synergized powerfully with frontline antimalarial drugs—artemisinin and chloroquine—lowering their IC₅₀ values by approximately 15-fold and 8-fold, respectively, thereby enhancing their efficacy (23). Moreover, inhibition of PfRad51 combined with Hsp90 blockers leads to severe DNA damage and reduced parasite viability,

emphasizing its druggable potential (43). Collectively, these findings provided the first genetic evidence that Rad51-driven HR is essential for parasite survival, and demonstrate that perturbing Rad51 function can severely impair malaria pathogenesis.

The mechanism of mitochondrial DNA repair and maintenance in *P. falciparum* was also revealed in a study that highlights nucleus-encoded proteins PfRad51 and PfBLM as essential players in repairing mitochondrial double-strand breaks (DSBs) (40). It was demonstrated that both proteins localize to the parasite's mitochondria during the schizont stage, a period highly susceptible to DNA damage due to metabolic stress, and are recruited to the mitochondrial genome in response to DSBs (40). This recruitment coincided with DNA repair kinetics, and it was seen that subsequent disappearance of these proteins mirrors completion of repair (40). Crucially, inhibition of PfRad51 and PfBLM specifically disrupts the repair of UV-induced double-strand breaks within mitochondrial DNA, whereas their overexpression results in a faster repair response (40). Given the vital role of the mitochondrion for parasite survival—and the absence of intrinsic repair machinery within its genome presents the fact that mitochondrial HR pathway could be used as a promising target for novel antimalarial strategies.

The functional role of the RecQ helicase, PfBLM, in the DNA double-strand break repair machinery of *P. falciparum* has been comprehensively characterized (44). It is established that PfBLM physically interacts with PfRad51 and PfMRE11—key recombination and DNA damage response proteins—and that its expression is significantly upregulated in response to genotoxic stress (44). Using yeast complementation assays, it was demonstrated that wild-type PfBLM could rescue the DNA damage sensitivity of an *S. cerevisiae* Δ sgs1 mutant, whereas a helicase-dead variant (Pfblm^{K83R}) failed to do so, underscoring the functional significance of its ATP-dependent helicase activity (44). Supporting its pivotal role in the DNA repair pathway, overexpression of PfBLM was observed to increase parasite tolerance to DNA-damaging agents, thereby improving overall survival (44). Importantly, the small-molecule RecQ helicase

inhibitor ML216 inhibited DSB repair in *P. falciparum*, rendering parasites markedly more sensitive to artemisinin (ART) and producing synergistic effects—increasing ART efficacy in both the drug-sensitive strain and the multidrug-resistant strain (44).

In a separate study, a novel apicoplast-targeted [4Fe–4S]-containing exonuclease (PfExo) with a unique low-complexity region (LCR) that interacts with PfPrex and broadens substrate recognition was identified recently (45). Functional and comparative analyses revealed that the LCR enhances DNA binding and nuclease versatility, and knockout studies in *P. berghei* established the enzyme's essentiality for parasite survival (45). PfExo thus represents the likely flap endonuclease and RNase H required for apicoplast DNA replication and maintenance. Its role in the HR pathway of the parasite could be studied further.

Table 1.2 lists all key conserved eukaryotic effector proteins of the HR pathway and highlights whether these have been previously identified and characterized in *P. falciparum* or not. In protozoan parasites such as *P. falciparum*, HR machinery is also central to survival, not only for DNA repair but also for generating diversity in virulence gene families such as *var* genes, which mediate immune evasion (46). All these findings discussed in this section suggest that the HR pathway could serve as a potential therapeutic target. Collectively, all these findings reveal the potential therapeutic usage of HR-mediated DNA DSB repair as a valuable target to sensitize parasites and to curb malaria.

HR Protein	Protein Function	<i>Plasmodium</i> Ortholog
Mre11	Complex that senses the DSB	Identified & characterized
Exo1	Performs 3' end resection	Identified & characterized
RPA	Binds ssDNA, protects, keeps it unfolded	Identified & characterized
Rad51	Key Recombinase, Strand Exchange	Identified & characterized
Rad52	Mediates complimentary strand annealing	Unidentified
Rad54	Facilitates sequence alignment and D-loop formation	Identified & characterized
Rad55-Rad57	Stabilizes Rad51-nucleofilament	Unidentified
Srs2	Anti-recombinase	Identified
DNA Polymerase	Performs DNA synthesis to fill the gap	Identified & characterized
Blm/Wrn	Resolves the Double Holliday junction	Identified & characterized

Table 1.2: List of all conserved eukaryotic HR proteins along with their functions.

1.3.3 The Research Gap.

The finding that inhibition of PfRad51—the principal recombinase of the homologous recombination (HR) pathway in *P. falciparum*—can disrupt DNA repair processes and consequently reduce parasite burden in the human host holds significant scientific and therapeutic importance. Nevertheless, a critical research gap remains. Specifically, the feasibility of targeting PfRad51 as an antimalarial drug candidate is limited by its considerable sequence homology with its human ortholog, HsRad51, with which it shares approximately 61% identity. This high degree of similarity and identity raises the concern of host toxicity, as pharmacological agents directed against PfRad51 would also interfere with the function of HsRad51, an essential protein for genome maintenance in human cells. Given this limitation, future drug discovery efforts should prioritize the identification of *Plasmodium*-specific proteins, or proteins that exhibit reduced homology with their human counterparts, but that remain indispensable for HR-mediated DNA repair and parasite survival. Such parasite-specific targets would provide a more selective and safer therapeutic avenue for antimalarial drug development, minimizing off-target effects while exploiting a pathway that is crucial for the parasite's proliferation and persistence in its host.

1.4 Random Walk and Restart (RWR) identified a conserved novel DNA repair protein (PfNdr).

In a quest to identify such a *Plasmodium*-specific drug target, a Random Walk with Restart (RWR) analysis revealed a novel DNA repair candidate in *P. falciparum* and this protein was named as PfNdr (Novel DNA Repair protein). A protein–protein interaction (PPI) network was constructed using known DNA repair genes as seed nodes and a previously uncharacterized gene, *PF3D7_0419300/PfNDR*, emerged as a top-ranking candidate within the network, while most other high-scoring genes were already characterized or corresponded to previously annotated DNA repair factors (47). PfNdr (PF3D7_0419300) is predicted to functionally associate with the DNA repair machinery in *P. falciparum*. This bioinformatic analyses was performed in a collaboration with D. Das and A. Roy, TCS Hyderabad (Abhilasha Gahlawat *et al*, unpublished data). We further performed a multiple sequence alignment (MSA) by taking the sequence of PfNdr and its orthologs from other *Plasmodium* species. Our phylogenetic analysis revealed that PfNdr is evolutionarily conserved across multiple *Plasmodium* species, suggesting a functionally important and possibly conserved role in parasite biology. Hence, from this preliminary data, we hypothesize that PfNdr might have a role in the homologous recombination mediated DNA DSB repair of malaria parasites.

1.5 Significance of the study.

Malaria continues to exert a profound impact on both global public health and economic stability, despite sustained efforts over several decades directed towards vaccine development and antimalarial drug discovery. The absence of a fully effective malaria vaccine, coupled with the widespread emergence of resistance to all currently available therapeutic agents, underscores the urgent need to identify novel intervention strategies. We also discussed the limitations associated with targeting key HR-specific DNA repair proteins in *P. falciparum*. In this context, the findings of this study report the identification and characterization of a novel DNA repair protein in *P. falciparum*, PfNdr. Functional analyses reveal that PfNdr operates within the homologous recombination (HR) pathway, alongside PfRad51, and is involved in the repair of DNA double-strand breaks through HR-mediated mechanisms. Most importantly, PfNdr exhibits only 14.45% sequence identity with its human ortholog, HsXRCC3, in contrast to the 61% sequence identity observed between PfRad51 and HsRad51. This low degree of homology significantly mitigates the risk of host toxicity, making PfNdr a more attractive candidate for therapeutic targeting. Furthermore, superimposition of PfNdr on HsXRCC3 using molecular docking illustrates the presence of multiple distinct and targetable regions on PfNdr. While human XRCC3 has been implicated in both HR and DNA replication, similar multifunctional roles of PfNdr remain to be elucidated. Nevertheless, its predicted interactions with the mitochondrial polymerase, PfPolG, and DNA topoisomerases suggest that PfNdr may also contribute to mitochondrial DNA maintenance and other critical cellular processes. These multifaceted roles reinforce the potential of PfNdr as a promising and parasite-specific druggable target, offering new opportunities for the development of effective antimalarial interventions.

1.6 Objectives of the study.

The increasing prevalence of drug resistance in *Plasmodium falciparum* has created an urgent need for the discovery of novel therapeutic targets to combat malaria. Among the pathways of interest, proteins involved in homologous recombination (HR)-mediated DNA repair have been investigated as potential antimalarial drug targets. However, their high sequence similarity to human orthologs significantly limits their clinical applicability, as inhibition of such proteins risks host toxicity. To address this limitation, the present study focuses on the identification and characterization of a novel *Plasmodium*-specific DNA repair protein, designated PfNdr, which exhibits minimal sequence identity with its human ortholog. Given that HR constitutes the predominant DNA repair mechanism in *P. falciparum*, elucidating the role of PfNdr within this pathway may provide a promising avenue for therapeutic intervention.

In this study, we investigated the function of PfNdr in the HR-mediated DNA repair pathway of *P. falciparum*. Specifically, we quantified its stage-specific expression across the parasite's life cycle, examined its interactions with other known HR proteins, and assessed how exposure to exogenous DNA-damaging stress influences its upregulation and localization. Additionally, we explored the impact of PfNdr overexpression on the repair kinetics of the parasite to determine its functional contribution to DNA repair processes. Finally, we conducted comparative analyses to identify the putative functional ortholog of PfNdr in yeast, thereby providing further insight into its evolutionary conservation and potential mechanistic role. Collectively, these investigations establish PfNdr as a novel and parasite-specific candidate for antimalarial drug development.

1.7 Aims of this Ph.D. work.

1.7.1 To establish if PfNdr has a role in DNA repair of malaria parasites:

- Interaction studies of PfNdr with known DNA repair proteins using Yeast-two-hybrid assays
- Stage-specific expression of PfNdr in the parasite both at the transcript and the protein level
- Transcript and protein expression profiling of PfNdr upon exogenous DNA damage
- Localization studies of PfNdr upon DNA damage using immunofluorescence assays
- Effect of over-expression of PfNdr on the DNA repair of the parasite by quantifying the DNA repair kinetics post exogenous DNA damage

1.7.2 Deciphering the biological function of PfNdr in HR-mediated DNA repair:

- Yeast complementation studies revealing the functional ortholog of PfNdr in yeast
- DNA repair kinetics assay revealing that PfNdr is a high copy suppressor of PfRad51 inhibition in *P. falciparum*

Chapter 2

Materials and Methods

2.1 Recombinant DNA methods:

2.1.1 Bacterial Competent cell preparation:

An isogenic colony of bacteria was inoculated in the 5mL LB medium having the suitable antibiotic selection and in a shaker incubator, they were cultivated overnight at 200 rpm rotation and 37°C temperature. The next day, secondary inoculation was given in a 50mL LB medium containing the suitable antibiotic selection. The cells were grown till the optical density at 600nm reached 0.5. Cells were harvested by centrifuging the culture at 8000 rpm for 10 minutes at 4°C. The bacterial pellet was washed by gently re-suspending it in 12.5mL of ice-cold 0.1 M CaCl₂ solution. Then cells were pelleted down by centrifuging the suspension at 8000 rpm for 10 minutes at 4°C. Later the cells were again resuspended in 12.5mL of ice-cold 0.1 M CaCl₂ solution followed by 4-8 hours of incubation on ice. After the incubation, competent cells were harvested by centrifuging the suspension at 8000 rpm for 10 minutes at 4°C. The resulted cell pellet was re-suspended gently in 2.15mL ice-cold 0.1 M CaCl₂ solution and 100µL 100% glycerol. Finally, 100µL of competent bacterial cell suspension were aliquoted into the pre-chilled microfuge tube and preserved at -80°C.

2.1.2 Bacterial Transformation:

Vials of competent bacterial cells were obtained from -80°C and thawed on ice for 15 minutes for bacterial transformation. Plasmid DNA with a preferable amount of 25-50 ng was laid over the cells and incubated on ice for the next 30 minutes. Cells were given heat shock at 42°C temperature after being incubated on ice, for a different duration depending upon cell type for example 30 seconds for Top10 cells 45 seconds for BL21 Rosetta cells, or 90 seconds for BL21 pLysS cells. Immediately after the heat shock cell were placed on ice for 1 minute and later 900µL of 37°C pre-warmed LB medium was added to the cells. Next, the cells were incubated at 37°C for 45 minutes to 1 hour. After this incubation, cells were pelleted down by applying brief spin at top centrifugal speed. Most of the supernatant was discarded leaving approximately 100µL medium for re-suspending the cells. Lastly, the suspension of transformed cells was

evenly spread over the LB agar plate having the suitable antibiotic selection and grown at 37°C for overnight (12- 16 hours).

2.1.3 Alkaline Lysis Method for Plasmid DNA Isolation:

An isogenic bacterial colony was inoculated into a 5-10mL LB medium containing suitable antibiotics and grown overnight in a shaker incubator at 200 rpm and 37°C temperature. The next day, the cells were harvested by centrifuging the culture at 4000 rpm for 10 minutes at room temperature. After the removal of supernatant media completely, the cell pellet was resuspended in 200µL solution 1 containing 25mM Tris-HCl pH 8 and 10mM EDTA pH 8. This cell suspension was transferred into a fresh 1.5mL microfuge tube. To this, 200µL of solution 2 which contains 0.2 mM NaOH and 1% SDS was added and then the samples were mixed thoroughly by inverting the tube multiple times until the cell suspension became viscous which ensures cell lysis. Next, 200µL solution 3 containing 3M NaOAc pH 5.2 and glacial acetic acid (GAA) was added and mixed very gently for avoiding mechanical shearing of DNA. This mixture was incubated on ice with intermittent mixing every 5 mins until while precipitates started appearing. This turbid solution was centrifuged at 12000 rpm for 10 minutes at room temperature. The resulted supernatant was transferred into a fresh microfuge tube and to this 400µL of absolute ethanol was added and samples were incubated at -80°C for 45 minutes. For precipitating the DNA, samples were centrifuged at 12000 rpm for 30 minutes at 4°C. The resulting pellet was washed with 70% ethanol and again centrifuged at 12000 rpm for 5 minutes at 4°C. The supernatant was discarded and pellets were air-dried for 20 minutes. The pellet was resuspended in 50µL 1X TE (10mM Tris-HCl pH 8 and 1 mM EDTA pH 8) and then treated with RNase by adding 5 µl of 10 mg/mL RNase stock solution. The samples were incubated in the 37°C water bath for 30 minutes. For PCIA treatment, the volume of samples were made up to 400µL using 1X TE to which an equal volume of PCIA solution containing Phenol: Chloroform: Isoamyl alcohol in 25:24:1 ratio was added. For homogenous mixing, the samples were vortexed for 3-5 minutes. To separate the aqueous layer from the organic layer the samples

were centrifuged at 12000 rpm for 15 minutes at room temperature. The resulting upper aqueous layer was carefully collected into a fresh microfuge tube and to this 2.2 volume absolute ethanol and 1/3 volume 3 M NaOAc solution was added and incubated at -80°C for 1-2 hours. The samples were centrifuged at 12000 rpm for 30 minutes at 4°C for precipitation of the purified plasmid DNA. The pellet was once washed with 500µL of 70 % ethanol followed by air-drying the pellet for 20 min. Finally, the DNA pellet was resuspended in 50µL of 1X TE solution and kept at -20°C for long time storage.

2.1.4 Site-Directed Mutagenesis:

The point mutation (D to A at the 194th position) was generated in *PfRAD51* by mutating the codon GAT to GCA using the splicing by overlap extension (SOE) PCR technique. The ORF of this gene was amplified in two fragments and the overlapping flaps of these fragments harboured the desired mutation. The homologous sequence of overlapping fragments allowed them to anneal and hence acted as the template while amplifying the full-length ORF of the *PfRAD51* gene. The details of primers which were used in generating this *Pfrad51*^{D194A} mutant are listed in table 2.3. The final full-length *Pfrad51* ORF with the desired mutation was cloned into the bacterial expression vector, pET28a and in pGAD-C1 for protein purification and yeast-two-hybrid analyses respectively. The final clones were sequenced in order to confirm the desired mutation.

2.2 PCR-Based Methods:

2.2.1 Real time PCR:

Applied Biosystems 7500 Fast Real Time PCR system was used for the Real time analysis. 10µL of reaction mixture was prepared which comprised of 0.5µL of the forward and reverse primers (concentration 10 pmole/µL) each, 5µL of SYBR premix (Takara), 2µL of Milli Q water and 2µL of the template. The fold change was calculated using Δ CT method. The mean values ($\pm$ SEM) from three independent experiments were plotted using Graph Pad Prism 6 software.

2.2.2 PCR-based method to quantify nuclear DNA repair kinetics:

Ring stage parasites were either untreated or pre-treated with a sub-lethal dose of the inhibitor B02 (1 μ M in DMSO) for repair kinetics assays. Untreated or B02 pre-treated parasites were irradiated with 100-J/m² UV light at the trophozoite stage. After DNA damage, the mock-treated and drug-treated parasites were cultured in the respective drug-containing medium for 36 hours. Samples were collected before and after UV treatment at every 6 hour interval. Genomic DNA was isolated from harvested parasites. An equal amount of DNA was used as a template to amplify long- and short-range fragments. Primer sets OMKB463 and OMKB464 and OSB94 and OSB95 were used to amplify the long-range PCR and short-range PCR products, respectively (44,48). Band intensities from the short-range PCR products were used to normalize the data. DNA damage was considered 0% in UV-untreated control and 100% in 0 hour post-UV treated sample. GraphPad Prism software was used to plot the percent residual damage at each time point. The mean band intensity $\pm$ standard error of the mean (SEM) are shown ($n = 3$).

2.3 Yeast Methods:

2.3.1 Yeast Competent Cell Preparation:

An isogenic colony of desired yeast strain was used for giving primary inoculation in 10mL YPD or YNB medium containing an appropriate amino acid mix. The yeast cells were grown in the incubator shaker at 200 rpm, 30°C for overnight. The next day, OD₆₀₀ of primary culture was measured, and based on the OD, the volume of overnight culture required for secondary inoculum was measured using the following formula:

The volume of primary culture required (mL) =

$$\frac{\text{The final volume of secondary culture (mL)} \times \text{desired OD}_{600} \text{ of secondary culture}}{\text{OD}_{600} \text{ of primary inoculum} \times 2^2}$$

The culture was incubated in the shaker incubator at 30°C, 200 rpm till the final OD reached 0.5-0.6. Cells were harvested by centrifuging the culture at 3500 rpm for 5 minutes at 4°C. The resultant supernatant was discarded and the cells were washed with 10mL sterilized Milli Q

water and centrifuged at 3500 rpm for 5 minutes at 4°C. The pelleted cells were re-suspended in 300µL LiOAc solution containing 1X TE and 1X LiOAc. 200µL of these competent yeast cells were used for each transformation.

2.3.2 Yeast Transformation:

In a sterile 2mL microfuge tube, 1-5µg of sample DNA along with 10µg of carried DNA (salmon sperm DNA) was added. Competent cells were gently layered over the DNA mix. 1.2mL PEG solution (containing 1mL 10X LiOAc, 1mL 10X TE, and 8 mL 50% PEG2000) was added to each tube, this was followed by 30 minutes incubation in a shaker incubator at 30°C, 200 rpm. Later the cells were subjected to heat shock at 42°C temperature for 15 minutes. Cells were then pelleted down by centrifuging the suspension at 10,000 rpm for 10 seconds. The cell pellet was re-suspended in 200µL 1X TE buffer and plated on the YNB agar plates with appropriate amino acid selection. The plates were incubated at 30°C for 3-5 days till the transformed colonies started appearing.

2.3.3 Yeast Two-Hybrid Assay:

A previously described procedure was used for yeast two-hybrid analyses (30). To score the interaction between the fusion proteins with activation domain and DNA binding domain, the plasmid constructs were transformed in PJ69-4a yeast cells and then assessed by growing 10-fold serial dilutions of the yeast cultures on synthetic complete agar lacking leucine, uracil, and histidine (SC-Ura-Leu-His) or lacking leucine, uracil, and adenine (SC-Ura-Leu-Ade) drop out plates for 3 to 4 days at 30°C. The DNA repair genes *PfNDR*, *Pfrad51(D194A)* and *ScRAD57* were cloned into the bait vector *pGBDU-C1* containing the *URA3* selection marker for fusing a gene to the *GAL4* DNA binding domain. Similarly, *PfRAD51*, *PfDMC1*, *PfACTIN*, *PfPOLG*, *Pfrad51(D194A)*, *PfTOPOIII* and *PfBLM* were cloned into the prey vector *pGAD-C1* containing *LEU2* selection marker for fusing a gene to the *GAL4* activation domain. The final constructs were transformed into a Y2H yeast strain PJ69A4, which resulted in the generation of yeast strains namely, AGY1 (co-transformed with *pGBDU-C1-PfNDR* and *pGAD-C1-*

PfRAD51), AGY2 (co-transformed with *pGBDU-CI-PfNDR* and *pGAD-CI-PfDMC1*), AGY3 (co-transformed with *pGBDU-CI-PfNDR* and *pGAD-CI-PfACTIN*), AGY4 (co-transformed with *pGBDU-CI-PfNDR* and *pGAD-CI-PfPOLG*), AGY5 (co-transformed with *pGBDU-CI-PfNDR* and *pGAD-CI-EMPTY*), AGY6 (co-transformed with *pGBDU-CI-EMPTY* and *pGAD-CI-Pfrad51(D194A)*), AGY7 (co-transformed with *pGBDU-CI-PfNDR* and *pGAD-CI-Pfrad51(D194A)*), AGY8 (co-transformed with *pGBDU-CI-Pfrad51(D194A)* and *pGAD-CI-TOPOIII*), AGY9 (co-transformed with *pGBDU-CI-Pfrad51(D194A)* and *pGAD-CI-PfBLM*), AGY10 (co-transformed with *pGBDU-CI-Pfrad51(D194A)* and *pGAD-CI-PfPOLG*), AGY11 (co-transformed with *pGBDU-CI-EMPTY* and *pGAD-CI-EMPTY*), AGY15 (co-transformed with *pGBDU-CI-PfNDR* and *pGAD-CI-ScRAD55*), AGY16 (co-transformed with *pGBDU-CI-ScRAD57* and *pGAD-CI-ScRAD55*) and AGY17 (co-transformed with *pGBDU-CI-EMPTY* and *pGAD-CI-ScRAD55*). The success of a single transformation was assessed on either Sc –Ura or Sc –Leu single dropout plates. Similarly, co-transformation of bait and prey vectors with/ without inserted gene was examined on Sc –Ura –Leu double dropout plates. Interaction between the test protein was determined by patching the doubly transformed yeast cells on triple dropout plates (Sc –Ura –Leu –His and Sc –Ura –Leu –Ade). The weak interaction was scored by spotting the equal number of doubly transformed yeast cells on Sc –Ura –Leu –His whereas strong interaction was scored on both Sc –Ura –Leu –His/Ade triple dropout plates. Self-activation of reporter gene promoter (*GAL4* promoter) was also determined by scoring the growth of co-transformed yeast strain harbouring empty bait and empty prey, empty prey and bait with protein of interest, and prey containing protein of interest with empty bait on triple dropout plates.

2.3.4 Protein isolation by TCA method:

Primary inoculum was given with a single colony of the required yeast strain in 5mL of appropriate growth medium and was grown overnight at 30°C. The yeast strain was grown at 25°C. Secondary inoculum was given next day in 20mL of growth medium with the primary

culture using the formula: Volume of inoculum = (Final volume of secondary culture $\times$ 0.5 OD) / (Initial OD $\times$ 2²). The secondary culture was grown till the OD₆₀₀ reaches 0.5 and cells were harvested by centrifugation at 3500 rpm, 4°C for 5 minutes. The cell pellet obtained was re-suspended in 500mL sterilized Milli-Q water and transferred to 2 mL centrifuge tube. At this point, the cells were either stored at -80°C or were right away used for the protein isolation. For protein isolation, cells are initially washed by re-suspending it in 500 μ L of 20% TCA and then centrifuged at 12000rpm, 4°C for 5 minutes. The cell pellet was then re-suspended in 200 μ L of 20% TCA and glass bead was added. The sample was vortexed for 10 minutes and placed on ice for 5 minutes rest. This step was repeated 2 more times. The supernatant was collected and transferred to fresh microfuge tube when glass beads settled. The remaining glass bead and cell mixture was washed with 200 μ L of 5% TCA by mixing thoroughly and then supernatant was collected after settling of the glass beads. This steps was repeated till the supernatant obtained after washing of glass beads became clear. The supernatant collected was centrifuged at 3000 rpm, RT for 10 minutes. The supernatant was then discarded and the precipitate was re-suspended in 90 μ L 1X SDS-PAGE loading buffer. 10 μ L of 1M DTT and 50 μ L of 1 M Tris pH 8.8 were added to the sample and was then boiled for 5 minutes and spun at top speed for 5 minutes.

2.3.5 MMS spotting assay:

All yeast strains were grown overnight in YPD medium at 30°C. The following day, secondary cultures were grown till 0.5 OD₆₀₀ at 30°C. MMS induced DNA damage sensitivity of yeast strains used in this study was assessed by spotting 10-fold serial dilutions of the yeast cultures on synthetic complete agar lacking histidine (SC–His) in absence (untreated) or presence of 0.05% MMS (which is added at the time of plate pouring) and cells were allowed to grow for 3 to 4 days at 30°C.

2.3.6 MMS return to growth assay:

The DNA damage sensitivity of yeast strains toward 0.05% MMS were tested according to the earlier used protocol (49). In brief, all strains were grown overnight in YPD medium at 30°C. Next day, secondary cultures were grown till 0.5 OD₆₀₀ at 30°C and were subjected to MMS (0.05%) for 2 hours. Subsequently, the MMS treated cells were washed with deionized water and about 1000 cells were spread on YPD-agar SC-His plates. The plates were incubated for 40 h at 30°C, and the colonies obtained for both treated and untreated conditions were counted. Further, the percentage survivability was calculated using the formula: % Survivability = [(Number of colonies obtained on treated plate) / (Number of colonies obtained on untreated plate)] × 100.

2.3.7 UV sensitivity assay:

Yeast strains were subjected to different doses of UV radiation and their susceptibility were compared as described earlier (49). Briefly, the cells were grown till 0.5 OD₆₀₀ at 30°C and approximately 1000 cells were spread on YPD-agar SC-His plates. The spread cells were subsequently exposed to 50 J/m², 100 J/m², and 150 J/m² of UV radiation, followed by incubation at 30°C for 40 h. Later, the percentage survivability was calculated at each dose of UV radiation.

2.4 Recombinant Protein purification:

For purifying the wild-type PfRad51 protein, the plasmid *pET101D-PfRAD51*, generated by MKB (42) was transformed into *E. coli* expression cell BL21 Rosetta. As reported earlier the maximum expression of recombinant PfRad51 protein was obtained by 1mM isopropylthiogalactoside (IPTG) induction given at 0.8 OD₆₀₀ for 4 hours at 37°C, 200 rpm. Likewise, the plasmid *pET28a-PfRad51^{D194A}* was transformed into BL21 Rosetta cells and recombinant protein was induced using 1mM IPTG (Sigma) given at 0.6 OD₆₀₀ for 4 hours at 37 °C, 200 rpm. After induction, cells were harvested by centrifuging the culture at 8000 rpm for 8 minutes at 4 °C and can be stored at -80 °C (optional). For purifying recombinant protein

with His tag following procedure was used: lysis buffer (containing 50 mM NaH₂PO₄, 200 mM NaCl, 20 mM Imidazole, 50% (v/v) glycerol, 20 mM β-mercaptoethanol, and 2% (v/v) Tween 20) was added to the cells according to the weight of the pellet (3 ml lysis buffer is required for 1 gram cell pellet). Lysozyme with the final concentration of 1 mg/ml was added to the cell suspension and incubated for 45 minutes on ice. PMSF was added to the samples and the samples were sonicated for approximately 20-30 minutes with 10 seconds pulse at 50% amplitude followed by 20 seconds rest on ice. This cell lysate was centrifuged at 10000 g for 45 minutes at 4°C. The supernatant was collected into the fresh tube and later mixed with the 50% (w/v) pre-equilibrated Ni-NTA bead slurry. For binding, this mixture was rotated for 1 hour at 15-20 rpm in the cold room. Beads were allowed to settle down for 1-2 hours and the loading flow-through was collected. Beads were first washed using washing buffer I (containing 50 mM NaH₂PO₄, 20 mM Imidazole, 50% (v/v) glycerol, 20 mM β-mercaptoethanol, and 2% (v/v) Tween 20) and washing buffer II (containing 50 mM NaH₂PO₄, 150 mM NaCl, 50 mM Imidazole, 50% (v/v) glycerol, 20 mM β-mercaptoethanol, and 2% (v/v) Tween 20). Later, for eluting the bound recombinant proteins elution buffer I (containing 50 mM NaH₂PO₄, 150 mM NaCl, 250 mM Imidazole, 30% (v/v) glycerol, 20 mM β-mercaptoethanol, and 2% (v/v) Tween 20) was added to the column and fractions were collected in fresh tubes. Similarly, beads were subjected to another round of elution using elution buffer II (containing 50 mM NaH₂PO₄, 150 mM NaCl, 400 mM Imidazole, 30% (v/v) glycerol, 20 mM β-mercaptoethanol, and 2% (v/v) Tween 20). All the fractions were analysed on SDS-PAGE for the presence of purified recombinant protein. Elution having the maximum amount of protein was subjected to dialysis in buffer containing 20 mM Tris-HCl pH 8, 1 mM dithiothreitol (DTT), 5% (v/v) glycerol. For purifying the PAP mutated PfRad51D^{194A} protein, the plasmid *pET23a-Pfrad51D194A* was used and same protocol was repeated.

2.5 Western blotting:

Protein from different sources such as purified recombinant protein or whole parasite lysate samples was separated on 10-12% SDS-PAGE followed by blot transfer. For blot transfer, PVDF membranes were first pre-treated with 100% methanol for 30 seconds followed by a quick wash with distilled water, and later the membrane was treated with transfer buffer (containing 5.86 grams of glycine, 11.64 grams of Tris base, and 0.75 grams of SDS) for 5 minutes. The semi-dry transfer method was employed for blot transfer at 240 mA constant current for 80 minutes. Followed by transfer, the blot was treated with 5% blocking buffer (containing 5 grams for skimmed milk in 100 mL 1X TBST buffer) for 2 hours on the rocker, at room temperature . Later, the blot was treated with primary antibody and incubated on the rocker overnight at 4°C. The next day, the blot was washed thrice using 1X TBST (containing 0.2 M Tris base, 9% NaCl pH of buffer adjusted to 7.6, and 0.1 % Tween 20) and later was probed with secondary antibody conjugated with HRP. The blot was then subjected to washing as described above. Followed by blot development using a chemiluminescent HRP substrate (SuperSignal West Pico PLUS; Thermo Scientific) and imaged by using a ChemiDoc Touch imaging system from BioRad. For quantifying, the protein band intensities ImageJ software was used. The primary antibodies used were rabbit anti-PfNdr (1:5000 dilution; Peptide antibody generated by KRIC), rabbit anti-PfRad51 (1:5000 dilution) (30), rabbit anti-GFP (1:5000; Abcam) and mouse anti-GAPDH (1:5000; Abcam). The horseradish peroxidase (HRP) conjugated secondary antibodies used in the study were anti-mouse (1:10000; Santa Cruz Biotechnology Inc.) and anti-rabbit (1:10000; Promega). The anti-PfNdr antibody was generated by selecting a unique peptide region with high-antigenicity on PfNdr protein (EASSFNKSKRKKLEFYDEK), and antibody was raised against the peptide in rabbit.

2.6 Methods in *Plasmodium falciparum* experiments:

2.6.1 Washing of Red Blood Cells (RBCs):

Blood was purchased from Sanjeevani blood bank for experimental use. 10mL of whole blood was aliquoted in 15mL centrifuge tube and subjected to centrifugation at 2500 rpm for 20 minutes at room temperature to separate RBCs. The top layer (serum) and intermediate buffy layer were aspirated carefully. The bottom layer containing enriched RBC was washed by resuspending the cells into an equal volume of cold incomplete RPMI medium and centrifuged at 2500 rpm for 20 minutes. Later, the supernatant was discarded and the above-mentioned washing steps were repeated twice. Finally, the washed RBCs were resuspended into an equal volume of cold incomplete RPMI medium to obtain 50% haematocrit and stored at 4°C.

2.6.2 Thawing of parasites:

A frozen vial of parasites was taken out from the liquid nitrogen storage tank and allowed to thaw by placing the tube in a 37°C water bath for 5 minutes with continuous monitoring. The vial was properly sterilized using 70% ethanol and its content was transferred into a 50mL centrifuge tube. To this, 200µL of solution I (12 % w/v NaCl) was added drop-wise with gentle mixing, and the cells were allowed to rest for 5 minutes. To this, solution II (1.6% w/v NaCl) was added in a drop-wise manner with intermittent gentle mixing. Later the suspension was centrifuged at 1000 for 10 minutes. The resultant supernatant was aspirated out without disturbing the cell pellet. The pellet was disturbed by gently mixing and to this, solution III (0.9% w/v NaCl, 0.2% w/v glucose) was added in a similar manner as described earlier. The cells were harvested by centrifuging the suspension at 1000 rpm for 10 minutes and the supernatant was discarded. Finally, the thawed parasite-infected RBCs were resuspended in 2 ml of complete medium. To this, 200µL of washed RBCs (50% haematocrit) was added and the culture was transferred to 6 well plates and grown in the candle jar placed within 37°C incubator.

2.6.3 *In vitro* parasite culture maintenance:

During the *P. falciparum* culture maintenance, the medium of the culture was preferably changed every day and a new subculture was given every alternative day (depending upon the donor culture parasitaemia). For changing the medium the upper layer medium from the plate was aspirated carefully without disturbing the settled cells (infected/ noninfected RBCs) and to this, 4mL fresh pre-warmed complete RPMI medium was added. The plate was shaken in the rotational motion to mix the cells with the added medium. For determining the parasitaemia of the culture, a blood smear was made on a clean slide which was then fixed with 100% methanol. The slide was next stained with Giemsa stain (Sigma), a nucleic acid stain specifically stains the parasites (infected RBCs). This was visualized under the oil immersion 100X lens of the compound microscope. For subculturing, the donor culture was first mixed well, and depending upon its parasitaemia, the required volume of donor culture was transferred to a fresh well of the plate to which 4mL of prewarmed complete RPMI medium and 0.5mL of washed blood was added. The plates were transferred into the candle jar placed inside the 37°C incubator.

2.6.4 Parasite Culture Synchronization by Sorbitol Method:

Parasite culture with more than 60% ring staged parasites with at least 4% parasitaemia in total was used for synchronization. The culture was transferred into 15mL centrifuge tube (per tube 10mL culture) and centrifuged at room temperature, 2500 rpm for 10 minutes. The resultant supernatant was aspirated out and to the cells, 1 ml of prewarmed sorbitol solution (5% w/v sorbitol) was added. Cells were re-suspended by brief vortexing and then incubated at 37°C (water bath) for 20 minutes with intermittent mixing. The volume in the tube was made up to 10mL using prewarmed in-complete media and centrifuged as mentioned above. The culture was washed thrice using in-complete media and finally, the cells were resuspended in prewarmed complete media and kept back in the candle jar at 37°C.

2.6.5 Protein preparation from the parasites:

The parasite pellet obtained after saponin lysis from 10-15 ml of the culture was re-suspended in 1X Laemmli buffer (63 mM Tris-HCl – pH6.8, 10% glycerol, 0.005% Bromophenol blue, 0.1% 2- Mercaptoethanol). The sample was boiled in boiling water bath for 10 minutes and then centrifuged at 8000 rpm for 8 minutes. The protein quality was checked on SDS–PAGE.

2.6.6 RNA isolation from the parasites:

Total RNA was isolated following the protocol described elsewhere (90). Ten milliliters of synchronised culture was centrifuged at 1800 rpm for 3 minutes and supernatant was removed. Pre-warmed TRIzol was added to the pellet. For ring stage 10 pellet volume and for trophozoite and schizont 20 pellet volume of TRIzol was used. The sample was shaken to dissolve the clumps and then incubated at 37°C for 5 minutes. The sample was vortexed for 3 minutes after adding 0.2 TRIzol volume of chloroform and then allowed to stand for 3 minutes. To extract the aqueous layer, the sample was centrifuged at 4500 rpm, 4°C for 30 minutes. The aqueous layer obtained was transferred to new microfuge tube, 0.5 TRIzol volume of 2-propanol was added and then incubated overnight at 4°C. Next day, the sample was spun at 14000 rpm, 4°C for 30 minutes. One milliliter of 75 % ethanol was added to the pellet and mixed. The sample was pooled in single tube if multiple tube were used and centrifuged at 14000 rpm, 4°C for 30 minutes. The pellet obtained was air dried for 5 minutes and re-suspended in 30 µl nuclease free water. The quality of RNA was assessed by running it in FA agarose gel.

2.6.7 cDNA preparation:

The concentration of isolated RNA was measured by spectroscopic analysis using JASCO spectrophotometer EMC-709. Five microgram of total RNA was subjected to DNase (Fermentas) treatment in order to remove the genomic DNA contamination. PCR analysis was performed without cDNA preparation (-RT) to confirm the complete removal of genomic DNA prior to cDNA synthesis. For cDNA synthesis, 1 µg of RNA was reverse transcribed with oligo

dT primer (Sigma) using Omniscript reverse transcriptase. The synthesized cDNA was further subjected to PCR analysis.

2.6.8 Transfection in *P. falciparum*:

A cycle prior to the transfection the synchronized parasite culture was fed with fresh blood while maintaining the parasitaemia at 2%. 100 µg of the desired plasmid was resuspended in 50µL of cold cytomix solution (containing 10 mM K₂HPO₄ pH 7.6, 120 mM KCl, 0.15 mM CaCl₂, 25 mM HEPES pH 7.6, 2 mM EGTA pH 7.6, and 5 mM MgCl₂) and incubated at 4°C overnight a day before transfection. On the day of transfection, 10 ml parasite culture with 8% parasitaemia at the early ring stage (6-10 hpi) was centrifuged at room temperature, 2500 rpm for 10 minutes. The harvested cells were washed twice with pre-warmed cytomix solution and later the cells were resuspended in 1250µL of cytomix. From this, 350µL of the cell suspension was added to the tube containing 50µL of DNA and mixed gently. This mixture was then transferred to an electroporator cuvette slowly by avoiding air-bubble formation. The cuvette was then placed carefully into the Bio-Rad Gene Pulsar, by aligning the metallic side of the cuvette with the direction of electrodes. The pulse was given at 0.31kV, 950µF, infinite resistance. Immediately after the electroporation, the content was transferred to the culture plate containing 3mL of pre-warmed complete medium. The remaining cells from the cuvette were collected by washing the cuvette multiple times with 1mL of ice-cold complete medium. To this, 100µL of fresh blood (50% haematocrit) was added and incubated at 37°C within the candle jar. After 6 hours, medium of the transfected culture was changed. Thereafter every day the media was changed and expansion of the culture was done once parasitaemia reaches 2%. The culture was maintained in pyrimethamine drug medium once the parasitaemia reaches 4-6% for allowing selective growth of transfectant over the non-transfectant parasite.

2.6.9 Genomic DNA isolation from *P. falciparum*:

Genomic DNA was isolated from 10mL parasite culture with at least 4% parasitaemia at different time points pre or post UV exposure. Parasites were released from RBCs by saponin

lysis and the parasite pellet was resuspended in 75 μ L of sterile Milli-Q water and to this 25 μ L of lysis buffer (containing 10 mM Tris-HCl pH8, 20 mM EDTA pH8, 0.5% SDS, and 0.1 mg proteinase K) was added. This mixture was thoroughly mixed by vortexing for 5 minutes at every 30 minutes interval during 3 hours of incubation at 37°C. The resulting lysed parasite was subjected to PCIA treatment to separate the aqueous layer. This top aqueous layer was collected in the fresh microfuge tube and subjected to RNase treatment for 30 minutes at 37°C. Later the samples were again treated with PCIA solution and centrifuged at top speed for 15 minutes to separate the aqueous layer which was collected in the sterile tube. To this 2.2 volume of 100% ethanol and 1/3 volume of 3M NaOAc pH 5.2 was added and the samples were incubated at -80°C overnight. The next day DNA was precipitated by centrifuging the samples at 12000 rpm for 30 minutes at 4°C which was followed by 70% ethanol wash of the DNA pellet and later the pellet was air-dried for 20 minutes. Finally, the isolated DNA was resuspended in 30 μ L 1X TE buffer and stored at -20°C refrigerator.

2.7 DNA damage induction:

2.6.1 DSB induction using UV:

To create random DSBs in the *P. falciparum* genome, tightly synchronized trophozoites (24 hours post-invasion) staged parasite with 2% parasitaemia (volume of culture varies depending upon the assay type) were used. At the time of treatment, maximum volume of medium from the wells containing parasite culture was aspirated and immediately the cells were exposed to UV light at a dose of 100J/m² (0.01J/cm²) using BIO-LINK® crosslinker (BLX). After the exposure, prewarmed complete medium was added and the culture was harvested at different time points and processed according to the assay.

2.6.2 DNA damage by methyl methane sulfonate (MMS):

The exogenous DNA damage stress was given to 150-200 ml synchronized schizonts (40 hpi). For inducing the DNA damage, 20 mL parasite culture was transferred into 50 mL centrifuge tubes and harvested at 2500 rpm for 10 minutes at room temperature . To each tube 20 mL

complete medium containing 0.05% MMS (i.e. 10 μ L 1% MMS in 200 mL complete medium) was added. Then cells were gently resuspended in MMS containing complete medium and transferred back to the culture plates followed by 6 hours incubation at 37°C within the candle jar. After this incubation, the culture was harvested and subjected to saponin lysis to liberate the parasites and stored at -80°C.

2.8 Indirect Immunofluorescence assay:

IFAs of parasites were performed according to the protocol described elsewhere (23). Briefly, 3D7 parasite cultures were synchronized using 5% sorbitol and allowed to grow to late trophozoite/early schizont stages and divided into two parts. One part was treated with 0.05% MMS for 6 hours, and the other part remained untreated. Cold methanol-fixed slides were treated with anti-PfNdr antibody (1:100 dilution) followed by incubation with anti-rabbit IgG (FITC-conjugated) (1:200 dilution). Parasite nuclei were stained with DAPI (10 μ g/ml) and visualized using a laser-scanning confocal microscope (Carl Zeiss). Parasite samples not treated with MMS acted as the “no DNA damage control.” The Pearson correlation coefficient (PCC) was calculated for immunofluorescence antibody (IFA) images using the JaCoP plugin of ImageJ (v.1.52s) software. GraphPad Prism software was used to plot the PCC values.

2.9 *In vitro* protein pull-down/co-purification assay.

For the pull down of PfRad51-interacting proteins from the parasite, recombinant His₆-tagged PfRad51 was purified from *E. coli* Rosetta (DE3) cells harbouring pET101D:*PfRAD51* as described previously (30). Soluble parasite lysates were prepared by resuspending a pellet of 0.05% MMS (methyl methane sulfonate) treated or untreated *P. falciparum* 3D7 cultures in lysis buffer (from Thermo Scientific Co-Immunoprecipitation Kit). Ice cold lysis buffer containing protease inhibitors was added to parasite pellet in a 10:1 v/w ratio and incubated for 1 hour. Samples were centrifuged at 16,200 $\times$ g for 10 min at 4°C. Parasite extracts were pre-cleared by passing thrice through equilibrated Ni-NTA resin (equilibration buffer: 20 mM Tris-HCl, 100 mM NaCl, pH 7.5). Such pre-cleared parasite extracts were used for the *in-vitro* pull

down experiment. 5µg of purified His₆-PfRad51 was allowed to bind to Ni-NTA beads pre-equilibrated with binding buffer. Unbound bait was removed by centrifugation at 900 × g for 5 min. For the pull-down, 200 µg of the pre-cleared lysate was allowed to bind to the immobilized His₆-PfRad51 in 200 µl of reaction buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1% NP40, 5 mM MgCl₂, 100 mM NaCl, 100 µM PMSF) for 12 hours at 4°C. The reaction mixture was then centrifuged at 900 × g for 5 min and the supernatant was discarded. The Ni-NTA beads were washed 5 times with the reaction buffer. The Ni-NTA bound proteins were then subjected to SDS-PAGE, followed by western blotting and probing with either anti-PfRad51 Ab (1:5000) (50) or anti-PfNdr Ab (1:5000).

2.10 Multiple Sequence Alignment and Phylogenetic Tree generation.

Protein sequences were retrieved from PlasmoDB, SGD or NCBI databases. All multiple-sequence alignments was performed using the DNA-STAR software. Phylogenetic trees were also constructed using the same software post their multiple sequence alignment.

Table 2.1: List of primers along with the nucleotide sequence and description of their usage in this work.

Primer Name	Primer Sequence	Description	Reference
OMK392	5' TCA GGA TCC ATG AAG TGT AGA CCA GTT GAC AC 3'	FP for the amplification of full length <i>PfNDR</i> with BamH1 site	This study
OMK393	5' CTA GTC GAC TTA TTT GTT TAT CTC TCC ACT TAT G 3'	RP for the amplification of full length <i>PfNDR</i> with Sal1 site	This study
OMKB426	5' ATT CTT CTT GTT CAA ATA CGG TC 3'	FP for semi-quantitative analysis of <i>PfNDR</i>	This study
OMKB17	5'ATC GGA TCC ATG AAA CAA GCA AAT ACA AAA G 3'	FP for the amplification of full length <i>PfRAD51</i> with BamH1 site	(23)
OMKB19	5' ATC GTC GAC TTT ATT TTT CCT CAT AAT CTG C 3'	RP for the amplification of full length <i>PfRAD51</i> with Sal1 site	(23)
OSB94	5'CTG TAA CAC ATA ATA GAT CCG AC 3'	FP used to amplify nuclear specific short-range PCR product in repair kinetics experiment and to normalize real time PCRs	(44)
OSB95	5' TTA ACC ATG GTT ATC ATC ATT ATT TC 3'	RP used to amplify nuclear specific short-range PCR product in repair kinetics experiment and to normalize real time PCRs	(44)
OMKB463	5' TCA GTC GAC ATG TTG AAT GAT ATG AAT GAT AAA AAAG 3'	FP used to amplify nuclear specific long-range PCR product in repair kinetics experiment	(44)
OMKB464	5' TCA GTC GAC TCA ACC TAT GTA ACC TTT ACA CTTC 3'	RP used to amplify nuclear specific long-range PCR product in repair kinetics experiment	(44)

OMKB777	5' ATC GGT ACC ATG AAG TGT AGA CCA GTT GAC 3'	FP used to amplify <i>PfNDR</i> for cloning into pARL vector with KpnI site	This study
OMKB778	5' ATC CCT AGG TTT GTT TAT CTC TCC ACT TAT GTG 3'	RP used to amplify <i>PfNDR</i> for cloning into pARL vector with AvrII site	This study
OMKB276	5' AAG AAT TGG GAC AAA CTC C 3'	RP GFP confirmatory primer	(40)
OMKB785	5' GTT TAC ATC CAA CGG CAT GTT TAA ATA ATA TAG C 3'	Mutation containing overlapping primer for PfRad51 (GAT codon for Aspartate is replaced by GCA codon for Alanine)	This study
OMKB786	5' AGC ATA TGC TAT ATT ATT TAA ACA TGC CGT TGG 3'	Mutation containing overlapping primer for PfRad51 with the reverse compliment of GCA codon	This study
OMKB787	5' TCA GGA TCC ATG CCT AGG GCC TTA TC 3'	FP used to amplify <i>ScRAD57</i> for cloning into pGBDU-C1 Y2H plasmid and pRS313 plasmid with BamH1 site	This study
OMKB788	5' CTA GTC GAC TCA GGC TGT TTC TAT TCC 3'	RP used to amplify <i>ScRAD57</i> for cloning into pGBDU-C1 Y2H plasmid and pRS313 plasmid with SalI site	This study
OMKB 932	5' TCA GGA TCC ATG TCG CTT GGT ATA CCA CTT TC 3'	FP used to amplify <i>ScRAD55</i> for cloning into pGAD-C1 Y2H plasmid with BamH1 site	This study
OMKB 933	5' CTA GTC GAC TTA ACC TTC ACT ATC ATA AAT TAT C 3'	FP used to amplify <i>ScRAD55</i> for cloning into pGAD-C1 Y2H plasmid with SalI site	This study

FP: Forward primer, RP: Reverse Primer

Table 2.2: List of yeast strains generated in this study and their phenotype description.

1.	AGY1	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/PfRad51</i>
2.	AGY2	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/PfDmc1</i>
3.	AGY3	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/PfActin</i>
4.	AGY4	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/PfPolG</i>
5.	AGY5	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/Empty</i>
6.	AGY6	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/Empty, pGADC1/PfRad51^{D194A}</i>
7.	AGY7	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/PfRad51^{D194A}</i>
8.	AGY8	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfRad51^{D194A}, pGADC1/PfTopoIII</i>
9.	AGY9	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 gal-ADE2, met::GAL7-lacZ, pGBDU-C1/PfRad51^{D194A}, pGADC1/PfBlm</i>

10.	AGY10	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 galA-ADE2, met::GAL7-lacZ, pGBDU-C1/PfRad51^{D194A}, pGADC1/PfPolG</i>
11.	AGY11	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 galA-ADE2, met::GAL7-lacZ, pGBDU-C1/Empty, pGADC1/Empty</i>
12.	AGY12	<i>MATa lue2-3, 112 trp 1-1, can1-100, ura3-1, ade2-1, his3-11,15, rad57Δ ::KAN^r pRS313/Empty</i>
13.	AGY13	<i>MATa lue2-3, 112 trp 1-1, can1-100, ura3-1, ade2-1, his3-11,15, rad57Δ ::KAN^r, pRS313/ScRad57</i>
14.	AGY14	<i>MATa lue2-3, 112 trp 1-1, can1-100, ura3-1, ade2-1, his3-11,15, rad57Δ ::KAN^r, , pRS313/PfNdr</i>
15.	AGY15	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 galA-ADE2, met::GAL7-lacZ, pGBDU-C1/PfNdr, pGADC1/ScRad55</i>
16.	AGY16	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 galA-ADE2, met::GAL7-lacZ, pGBDU-C1/ScRad57, pGADC1/ScRad55</i>
17.	AGY17	<i>MATa trp1-901, lue2-3, 112 ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3 galA-ADE2, met::GAL7-lacZ, pGBDU-C1/Empty, pGADC1/ScRad55</i>

Chapter 3

Results

To establish if PfNdr has a role in DNA repair of malaria parasites

3.1 PfNdr is an uncharacterized protein conserved across different *Plasmodium* species.

As discussed in the introduction, a Random Walk with Restart (RWR) algorithm on *P. falciparum* protein–protein interaction network seeded with known DNA repair genes identified a novel, previously uncharacterized putative protein, PfNdr (Novel DNA Repair protein). To further assess the evolutionary significance of PfNdr, we performed a multiple sequence alignment (MSA), which revealed strong conservation of PF3D7_0419300 (PfNdr) across six distinct *Plasmodium* species (Figure 3.1A). This was corroborated by a phylogenetic tree constructed using the DNA-STAR software, which further confirmed the evolutionary conservation of PfNdr across this genus (Figure 3.1B). These preliminary bioinformatic findings suggest that PfNdr is a conserved and potentially indispensable DNA repair protein in the *Plasmodium* lineage.

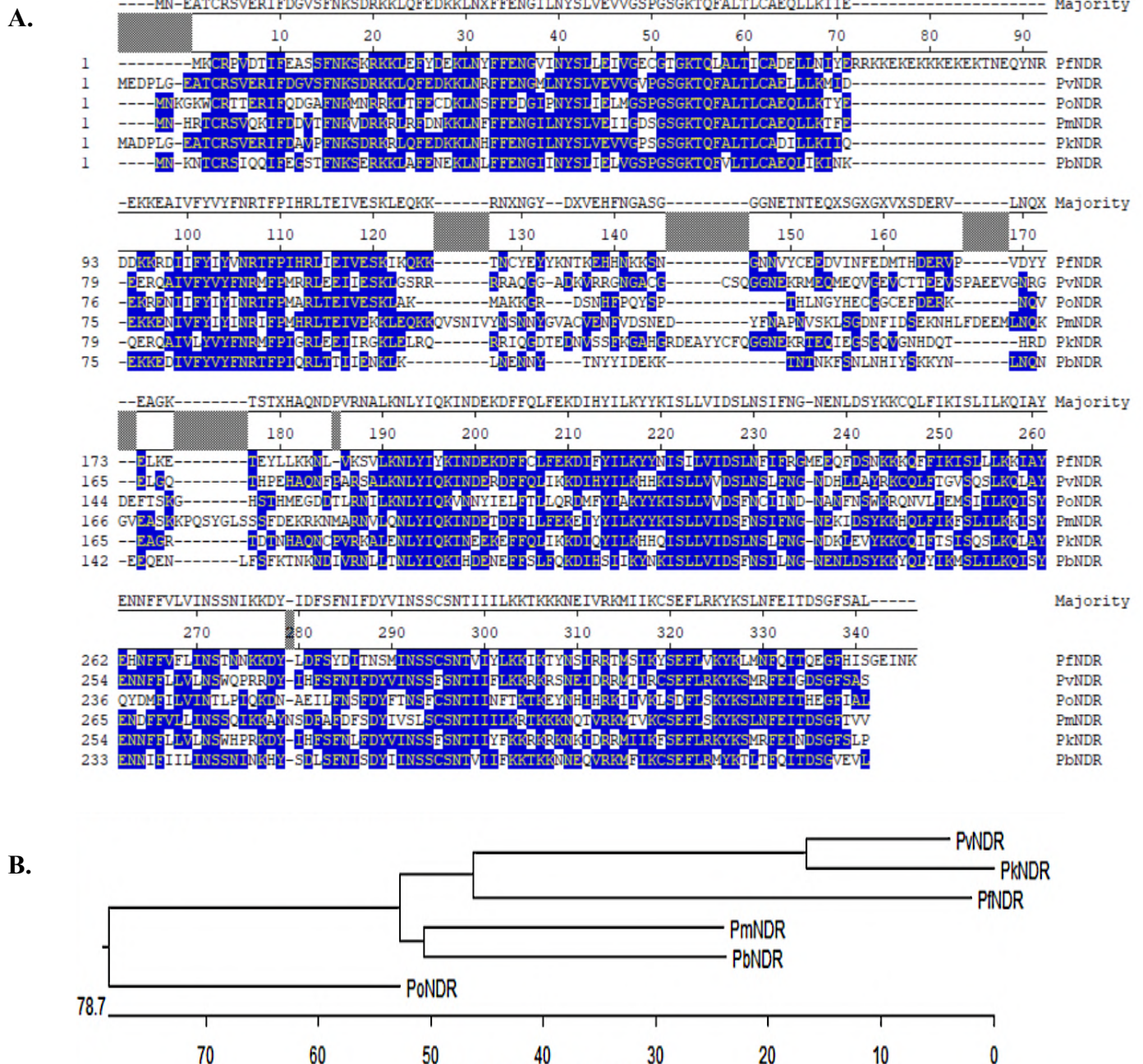


Figure 3.1. PfNdr is conserved among all *Plasmodium* species. (A) Multiple sequence alignment (MSA) of PfNdr from different species of the malaria parasite performed using DNA-STAR software. (B) Phylogenetic tree constructed using DNA-STAR software that shows the evolutionary conservation of PfNdr across different *Plasmodium* species.

3.2 PfNdr interacts with known DNA repair proteins.

Building upon the bioinformatic predictions generated via Random Walk with Restart (RWR) analysis, which indicated potential interactions between PfNdr and key DNA repair proteins, we sought to experimentally validate these protein–protein interactions using a yeast two-hybrid (Y2H) assay. Employing *S. cerevisiae* as a surrogate system, we tested the interaction of PfNdr with several known DNA repair proteins from *P. falciparum*. Our results revealed a robust interaction between PfNdr and PfRad51, the mitotic recombinase, and also with PfDmc1, the meiotic recombinase (Figure 3.2). A weak interaction was also observed with PfPolG, a mitochondrial DNA polymerase implicated in mitochondrial genome repair (Figure 3.2). PfRad51 and PfDmc1 are established mediators of homologous recombination during mitosis and meiosis, respectively, while PfPolG functions alongside PfRad51 and PfBlm in maintaining mitochondrial genome integrity (23,30,40,51).

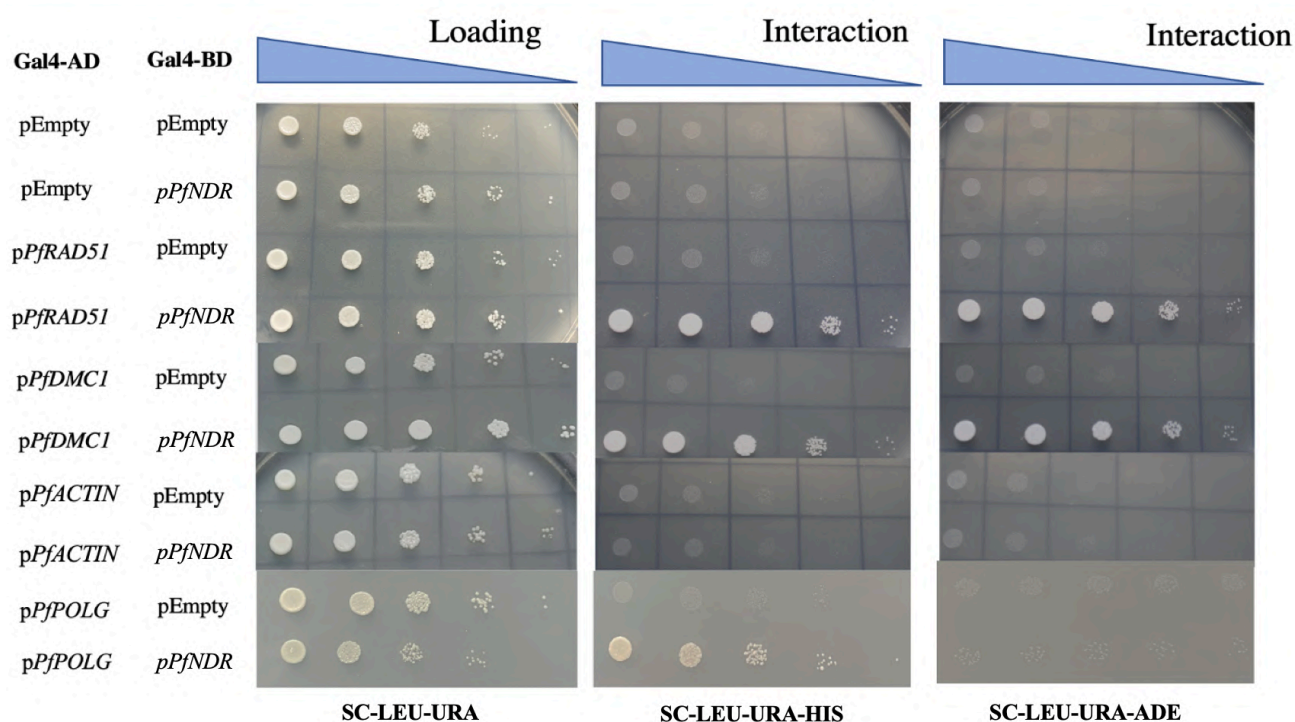


Figure 3.2. PfNdr interacts with key DNA repair proteins. Yeast two-hybrid analysis showing interactions between different parasite proteins. PfRad51, PfDmc1 and PfPolG were fused with the GAL4 activation domain (GAL4-AD) of the prey vector pGAD-C1. Likewise, PfNdr was fused with the GAL4 DNA binding domain (GAL4-BD) of the bait vector pGBDU-C1. Interactions of the fusion proteins were examined in PJ69-4A yeast strain containing *ADE2* and *HIS3* reporter genes under the *GAL4* promoter. An equal number of transformed yeast cells and their 10-fold serial dilutions were spotted on synthetic complete medium lacking uracil and leucine (Sc-Ura-Leu) agar plates to establish the expression of fusion proteins. Simultaneously, yeast cells were spotted on synthetic complete medium lacking uracil, leucine, and histidine (Sc-Ura-Leu-His) and synthetic complete medium lacking uracil, leucine, and adenine (Sc-Ura-Leu-Ade) triple dropout agar plates to assess the protein-protein interactions. Interaction between empty prey vector and PfNdr and between empty bait vector and PfRad51, PfDmc1 or PfPolG were used as experimental negative controls. Interaction between PfActin and PfNdr also acted as a negative control.

Inspired by a recent study in *Schizosaccharomyces pombe*, where SpRad51 was shown to contain a Protruding Acidic Patch (PAP) critical for interactions with its auxiliary recombination proteins, we investigated whether a similar motif exists on PfRad51 (52). In *S. pombe*, the PAP motif is located on the external surface of Rad51 and comprises of three conserved acidic residues (E205, E206, D209), whose mutation abrogates Rad51 interactions with its cofactors namely, SpRad55, SpRad57, and SpRad52 (52). A multiple sequence alignment (MSA) analysis of PfRad51 with other orthologs revealed a putative PAP-like region, represented by a single conserved acidic residue—Aspartic acid at the 194th position, D194 (Figure 3.3A). Using site-directed mutagenesis (SDM), we substituted D194 with a neutral alanine (PfRad51^{D194A}), and re-examined protein–protein interactions of this PAP-mutated PfRad51^{D194A} via Y2H (Figure 3.3B). As anticipated, the D194A mutant of PfRad51 exhibited a complete loss of interaction with PfNdr, PfTopo3, PfBlm, and PfPolG, unlike wild-type PfRad51, which retained strong interactions with these proteins on selective double dropout plates (Figure 3.3B). Western blot analysis using anti-PfRad51 antibody confirmed equivalent expression levels of both wild-type and mutant PfRad51 in the yeast strains used in this study (Figure 3.3C), ruling out expression differences as the cause of interaction loss. Furthermore, an independent *in vitro* co-purification assay was employed that corroborates the interaction between PfNdr and PfRad51. In this assay, PfRad51 bound to Ni-NTA beads was used to pull-down proteins from MMS-treated and untreated parasites. PfNdr was seen to be specifically pulled down from lysates treated with MMS and a lower signal was also seen in case of untreated parasite lysate (Figure 3.4, lane 1 and lane 3, respectively). No PfNdr signal was reported in the eluate from control beads alone (Figure 3.4, lane 5). Ni-NTA beads bound to recombinant PfRad51^{D194A} failed to pull-down PfNdr (Figure 3.4, lane 4). This assay further confirms the PfRad51-PfNdr interaction and also confirms that PfRad51^{D194A} mutant fails to interact with PfNdr, confirming the specificity of this interaction. Together, these findings validate the physical association between PfNdr and PfRad51 and highlight the functional

importance of the conserved acidic residue, D194, on PfRad51 in mediating PfRad51's interaction with its auxiliary partners in the HR pathway.

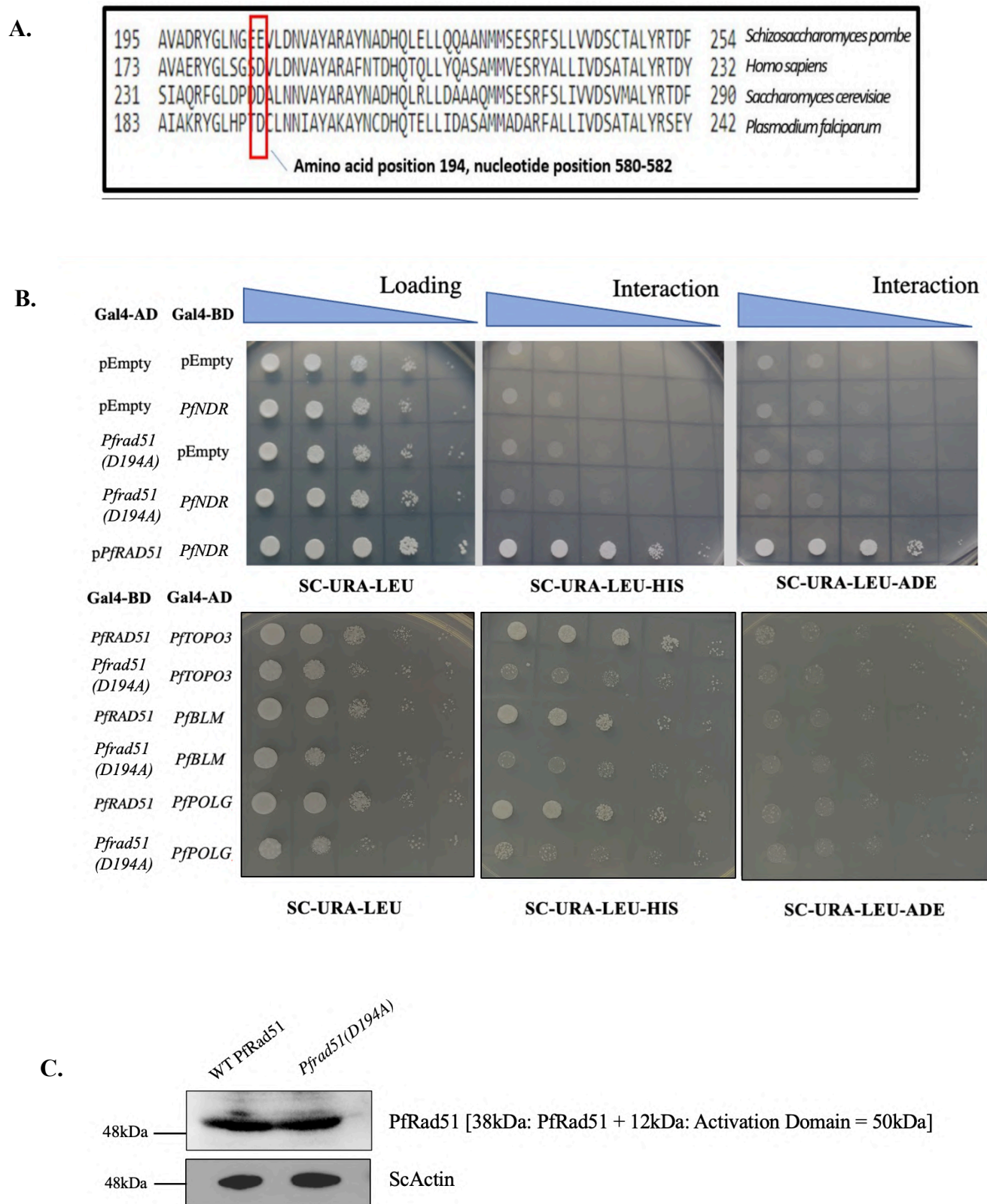


Figure 3.3. A PAP motif of *PfRad51* is crucial for protein-protein interactions. The PAP motif on *PfRad51* is composed of a single acidic amino acid, D194 and is critical for interaction with other DNA repair proteins. (A) Multiple sequence alignment showing conservation of the Protruding Acidic Patch (PAP) on Rad51 from different organisms. A single aspartic acid at the 194th position forms the PAP region on *PfRad51*. (B) Yeast-two-hybrid analysis showing that *PfRad51*^{D194A} fails to interact with key DNA repair proteins like *PfTopoIII*, *PfBlm* and *PfPolG* and also with *PfNdr*. *GAPDH* is used as normalizing control. (C) Western blotting confirms equal expression of both wild-type *PfRad51* and *PfRad51*^{D194A} in the yeast strains used in this assay.

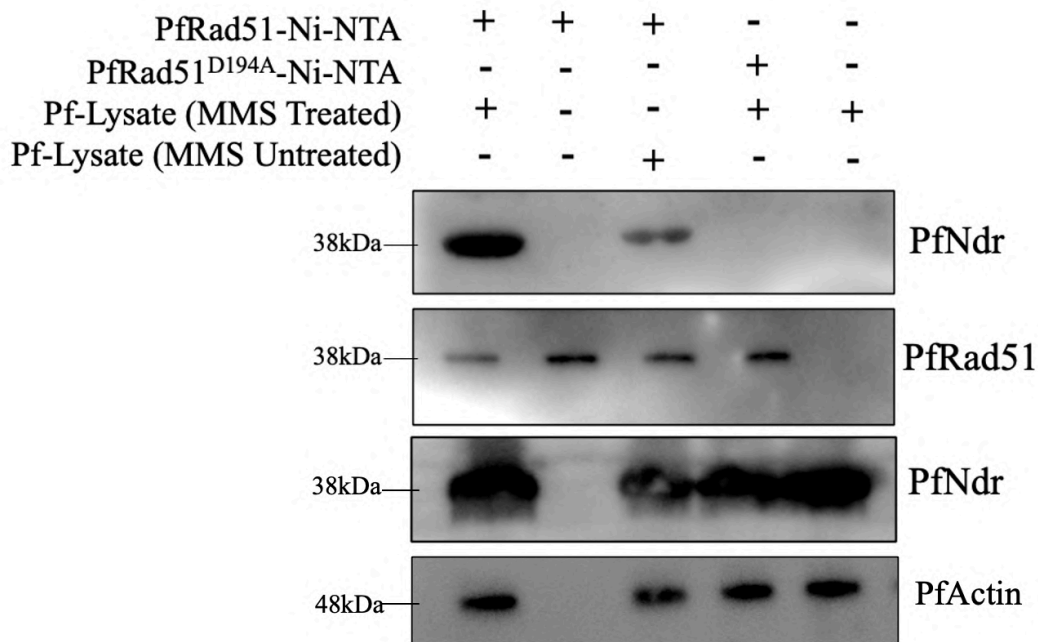


Figure 3.4. Specificity of PfRad51-PfNdr interaction. The PAP motif on PfRad51 is composed of a single acidic amino acid, D194 and is critical for interaction with other DNA repair proteins. Pull-down from lysates of MMS treated and untreated parasites using recombinant His6-tagged PfRad51 as bait. Western blots were probed with anti-PfNdr and anti-PfRad51 antibodies. PfNdr eluted with PfRad51 with a more prominent signal from MMS-treated parasites (lane 1) as compared to untreated parasites (lane 3). Lane 2 acts as a negative control where no lysate was passed through the beads. PfRad51^{D194A} fails to pull-down PfNdr (lane 4) and PfNdr was not pulled down by beads alone (lane 5).

3.3 PfNdr is expressed at the DNA damage prone stages of the parasite and gets upregulated upon exogenous DNA damage.

To investigate the stage-specific regulation of PfNdr during the intra-erythrocytic development, we analysed its expression at both the transcript and protein levels across the three asexual intra-erythrocytic stages of *P. falciparum*—ring, trophozoite, and schizont. A semi-quantitative real-time PCR analysis from cDNA extracted from three different stages of the parasite revealed that *PfNDR* transcript levels were markedly elevated during the trophozoite and schizont stages, with minimal expression observed during the ring stage (Figure 3.5A). Notably, these stages correspond to the mitotically active phase of the parasite, which aligns with the S-phase of the cell cycle and is known to be particularly susceptible to DNA damage. Transcript expression profile of *PfNDR* was seen to be congruent to that of *PfRAD51*, the canonical homologous recombination recombinase, suggesting a potential functional relationship (Figure 3.5A). Consistent with the transcript data, immunoblot analysis to quantify the basal stage-specific protein levels demonstrated that PfNdr protein levels peaked during the trophozoite and schizont stages, and this pattern also coincided with the protein expression of PfRad51 (Figure 3.5B and 3.5C). To further delineate the role of PfNdr in DNA damage response, we subjected the wild-type 3D7 malaria parasites to UV irradiation and assessed dose-dependent changes at the transcript level. In an unsynchronized parasite culture, both *PfNDR* and *PfRAD51* exhibited significant and parallel upregulation at increasing UV doses, confirming their involvement in the DNA damage response pathway (Figure 3.6A). Furthermore, when parasites at different intra-erythrocytic stages were exposed to a sub-lethal UV dose (100 J/m²), the most substantial transcript induction was observed during the trophozoite and schizont stages, with *PfNDR* and *PfRAD51* showing approximately 7-fold and 10-fold increases, respectively (Figure 3.6B). We also quantified the fold change of PfNdr at the protein level upon both UV (100J/m²) and 0.05% MMS-induced DNA damage (Figure 3.6C, 3.6D, 3.6E) and PfActin was used as the loading control. There was a 2-fold upregulation of PfNdr in both the DNA damaging conditions which

was quantified using Image J and the graph was plotted using GraphPad Prism (Figure 3.6E). These findings collectively support the hypothesis that PfNdr plays an important role in the DNA damage response machinery of *P. falciparum*, particularly during the replicative or mitotically active stages of the parasite's lifecycle which are also the DNA damage prone stages of the parasite.

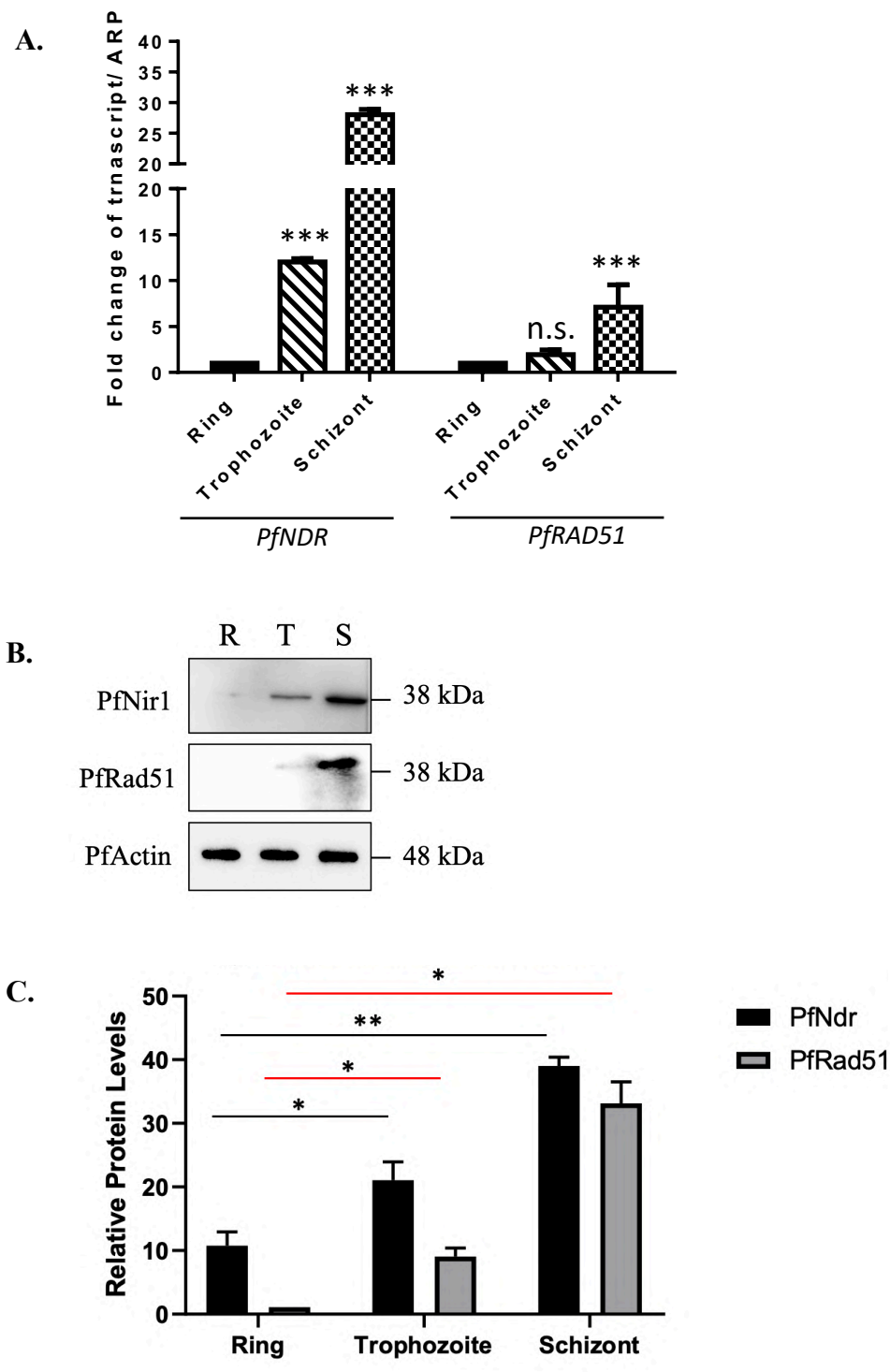


Figure 3.5. PfNdr exhibits maximum expression at the DNA damage prone stages of the parasite. (A) Relative abundances of *PfNDR* transcripts during the ring (12 h postinvasion [hpi]), trophozoite (24 hpi), and schizont (38 hpi) stages of the parasite quantified by real-time RT-PCR analysis. Data were normalized against *PfARP*. The mean values $\pm$ standard deviations (SD) from three independent experiments are plotted. (B) Stage-specific expression of the PfNdr protein. PfActin was used as the loading control. The position of molecular markers is indicated on the right. R,T and S represent the Ring, Trophozoite and Schizont stages of the parasite. (C) Quantification of Western blot data using ImageJ software from three independent experiments. Data were normalized against loading control PfActin. Each bar represents mean density $\pm$ SD ($n=3$). The P value was calculated using the two-tailed t test (* means a P value of <0.05 , ** means a P value of <0.01 , *** means a P value of <0.001 and N.S. means not significant).

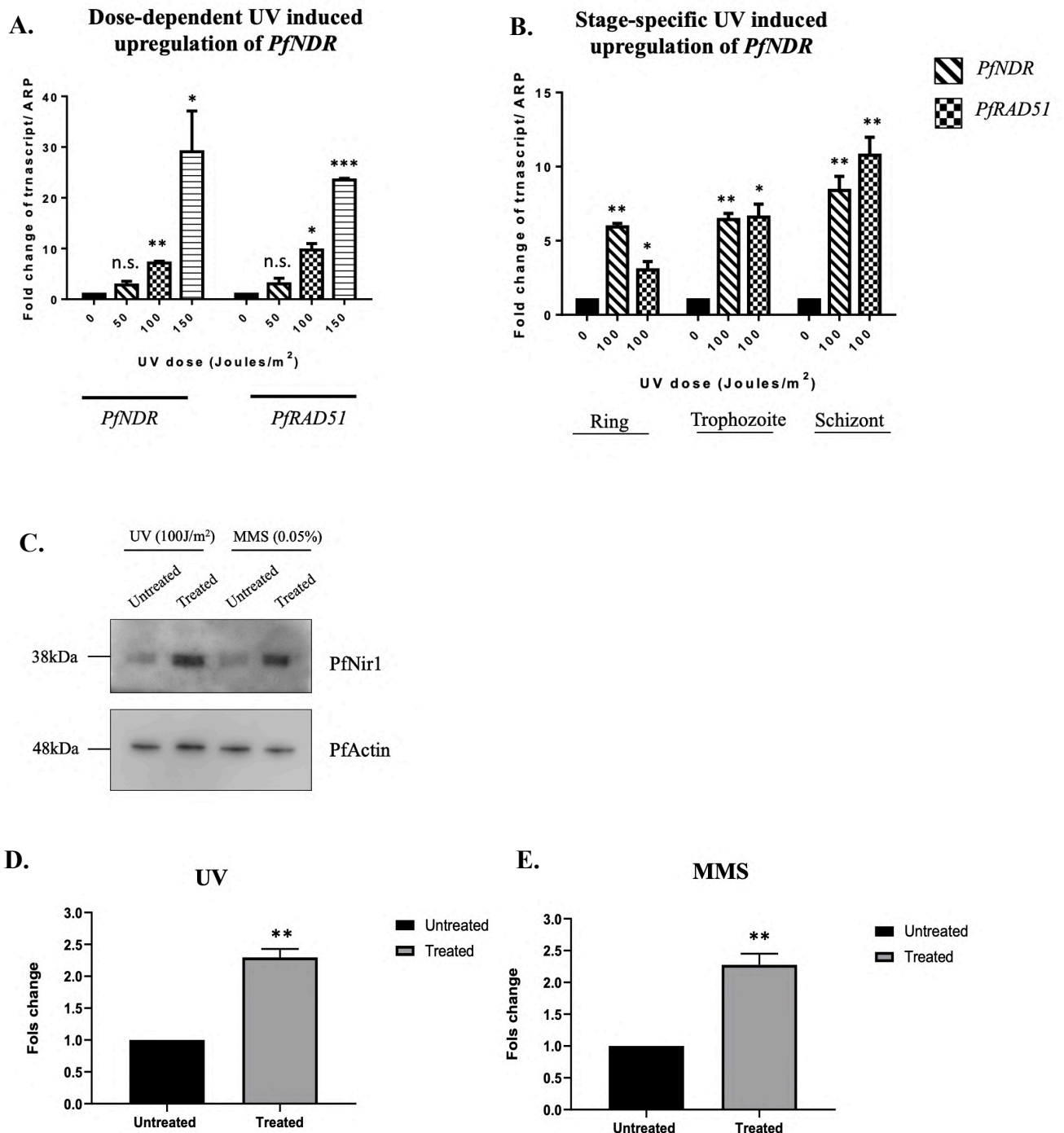


Figure 3.6. DNA damage-induced upregulation of *PfNdr* during intraerythrocytic developmental stages. (A) Unsynchronous *P. falciparum* *in vitro* cultures were treated with 0, 50, 100 and 150J/m² of UV irradiation and real-time RT-PCR on extracted RNA revealed the upregulation of both *PfNDR* and *PfRAD51* mRNA. *PfARP* transcripts were used as a loading control. (B) Synchronized *P. falciparum* *in vitro* cultures at the ring, trophozoite, and schizont stages were either untreated (U) or treated (T) with 100J/m² of UV irradiation and real-time RT-PCR on extracted RNA revealed the upregulation of *PfNDR* and *PfRAD51* mRNA during the trophozoite and schizont stages of the parasite upon DNA damage, but levels were not changed during the ring stage. *PfARP* transcripts were used as a loading control. (C) Western blots show that 100 100J/m² of UV irradiation and 0.05% MMS both induced the expression of *PfNdr* protein. *PfActin* acted as a loading control. The position of molecular markers is indicated on the left. (D) Quantification of Western blot data for UV treated parasites from three independent experiments using ImageJ software. Data were normalized against *PfActin*. (E) Quantification of Western blot data for MMS treated parasites from three independent experiments using ImageJ software. Data were normalized against *PfActin*. Each bar represents the mean band intensity $\pm$ SD ($n = 3$). The P value was calculated using the two-tailed t test (* means a P value of <0.05 , ** means a P value of <0.01 , *** means a P value of <0.001 , N.S. means non-significant).

3.4 PfNdr gets localized to the nucleus upon DNA damage.

To investigate the subcellular localization dynamics of PfNdr in response to genotoxic stress, we performed an immunofluorescence assay (IFA) under both normal and DNA-damaging conditions. To that end, parasites were probed with an anti-PfNdr peptide antibody, followed by detection with an anti-mouse Alexa Fluor-conjugated secondary antibody. Nuclear DNA was counterstained using DAPI. Under untreated conditions, PfNdr was seen to be primarily localized to the cytoplasm, as evident from minimal colocalization with the DAPI signal (Figure 3.7A). In contrast, upon exposure to 0.05% methyl methane sulfonate (MMS), a DNA-damaging agent, PfNdr displayed pronounced nuclear localization (Figure 3.7B). Our confocal IFA images and a quantitative fluorescence analysis using ImageJ reveals a significant increase in nuclear colocalization upon DNA damage by 0.05% MMS (Figure 3.7B), as reflected by the Pearson Correlation Coefficient (PCC) values (Figure 3.7C). Specifically, the mean PCC increase from 0.279 in untreated parasites to 0.663 in MMS-treated parasites (Figure 3.7C), indicates a robust translocation of PfNdr from the cytoplasm to the nucleus in response to DNA damage. These findings suggest that PfNdr is a DNA damage stress-responsive protein that potentially participates in nuclear DNA repair pathways under genotoxic stress conditions. Figure 3.8A gives the schematic for generation of these GFP-tagged PfNdr transgenic parasite. The confirmation of these GFP-tagged PfNdr transgenic parasites is also shown by a confirmatory western blot using GFP-antibody (Figure 3.8B) and also by an immunofluorescence assay performed using anti-GFP antibody (Figure 3.8C). The presence of green fluorescence in this assay confirms the presence of GFP-tagged PfNdr used in our study (Figure 3.8C).

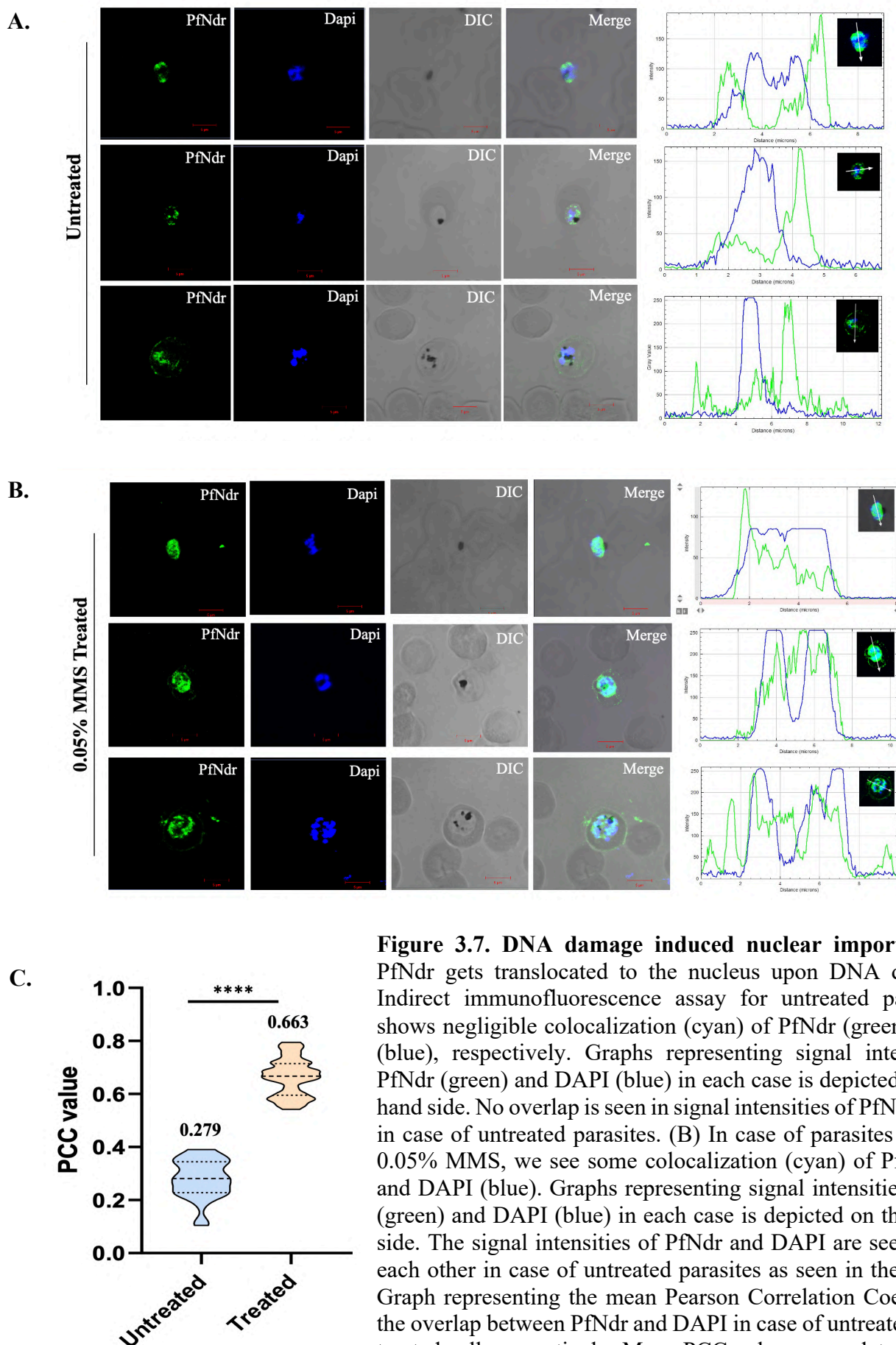


Figure 3.7. DNA damage induced nuclear import of PfNdr. PfNdr gets translocated to the nucleus upon DNA damage. (A) Indirect immunofluorescence assay for untreated parasites that shows negligible colocalization (cyan) of PfNdr (green) and DAPI (blue), respectively. Graphs representing signal intensities for PfNdr (green) and DAPI (blue) in each case is depicted on the right hand side. No overlap is seen in signal intensities of PfNdr and DAPI in case of untreated parasites. (B) In case of parasites treated with 0.05% MMS, we see some colocalization (cyan) of PfNdr (green) and DAPI (blue). Graphs representing signal intensities for PfNdr (green) and DAPI (blue) in each case is depicted on the right hand side. The signal intensities of PfNdr and DAPI are seen to overlap each other in case of untreated parasites as seen in the graphs. (C) Graph representing the mean Pearson Correlation Coefficients for the overlap between PfNdr and DAPI in case of untreated and MMS treated cell, respectively. Mean PCC values were determined from 30 individual cells from each of the IFA. *P* values were calculated using a two-tailed Student's *t* test (****, *P* < 0.0001).

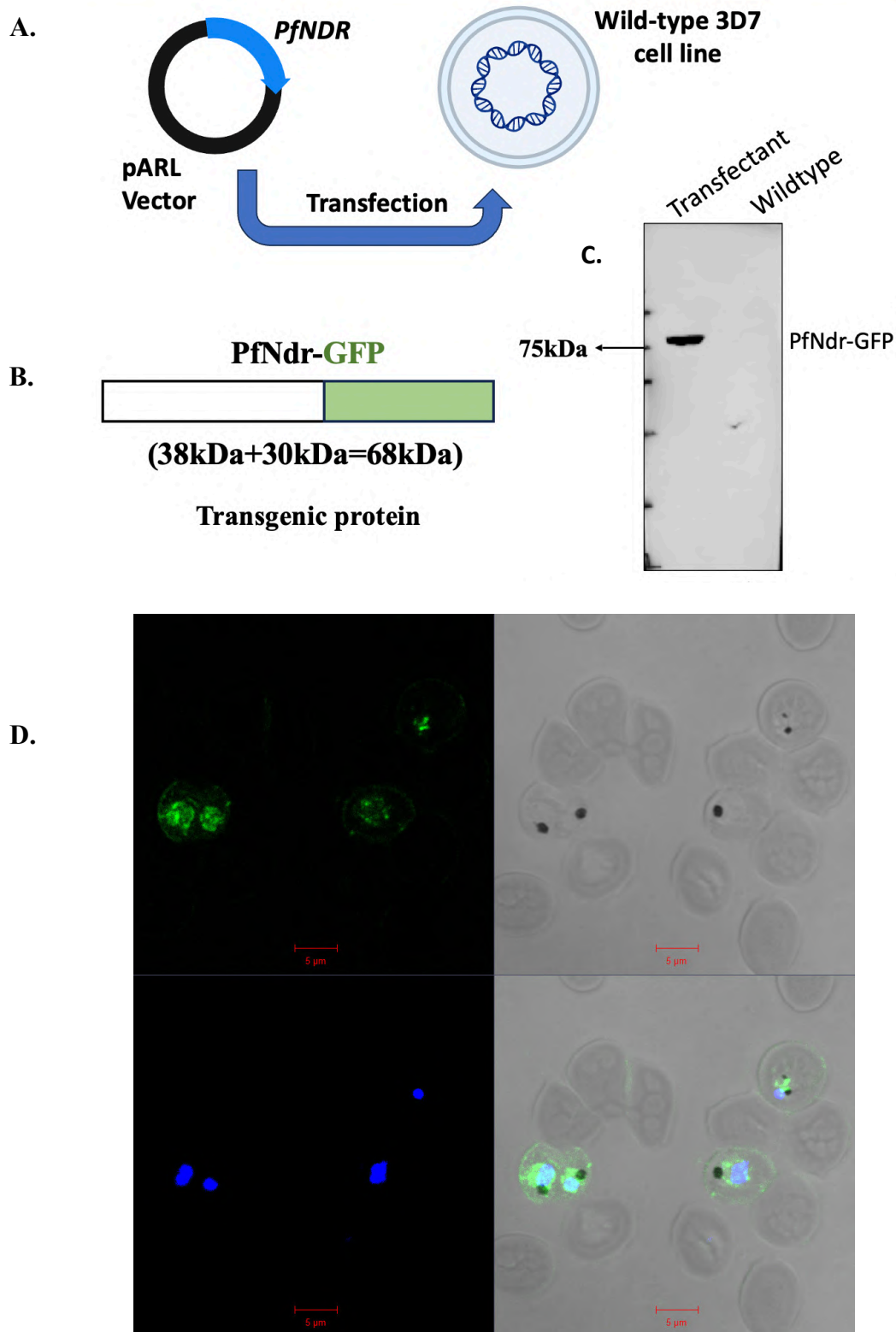


Figure 3.8. Confirmation of the transgenic GFP-tagged PfNdr parasites. (A) Schematic demonstrating the scheme of generation of GFP-tagged PfNdr parasites. (B) Schematic demonstrating the molecular size of transgenic PfNdr-GFP parasites. (C) Western blot demonstrating episomal expression of C-terminal GFP-tagged PfNdr in transfected parasite cell line (3D7, control parasite; T, transfected parasite). (D) Immunofluorescence assay using anti-GFP antibody, confirming the presence of GFP-tagged parasites.

3.5 PfNdr provides kinetic advantage to the DNA repair of malaria parasites.

To assess the functional role of PfNdr in DNA double-strand break (DSB) repair, we quantified the kinetics of DNA repair in three different *P. falciparum* strains: the wild-type 3D7 parasite line, a previously characterized PfRad51 dominant-negative (DN) mutant parasite line (PfRad51^{K193R}) (30), and a newly generated PfNdr-overexpression (OE) parasite line exogenously overexpressing GFP-tagged PfNdr. The PfRad51^{K193R} mutation acts in a dominant-negative fashion by impairing endogenous PfRad51 activity, thereby serving as a genetic background deficient in HR-mediated DNA repair (30). Immuno-blotting and IFA confirms the expression of GFP-tagged PfNdr transfectant cell line used in this study (Figure 3.8C). We employed a previously established PCR-based assay to monitor DNA repair by measuring the recovery of a long genomic fragment following UV-induced DSBs (44). This method is based on the principle that a longer region of the genome is likely to experience more UV-induced DNA double-strand breaks as compared to a shorter region and as a result, will not yield a PCR amplicon unless such DNA DSBs are repaired (Figure 3.9A). Conversely, a shorter fragment serves as an internal control as it is less likely to be disrupted (Figure 3.9A). In this assay, parasites from each strain were harvested at multiple time points (0, 6, 12, 18, 24, 30, and 36 hours) post-UV exposure, and the ratio of long to short fragment amplification was used to calculate the percent residual DNA damage over time for each of the three mentioned strains (Figure 3.9B). As anticipated and in consistent with previous reported findings, the PfRad51 DN strain exhibited persistent DNA damage with ~100% residual breaks even after 36 hours, reflecting complete impairment of DSB repair. In contrast, the wild-type 3D7 parasites displayed efficient repair, with most DNA lesions resolved by 24 hours post-treatment. Notably, the PfNdr-overexpressing strain demonstrated an accelerated repair kinetics, with a marked reduction in residual damage as early as 12 hours post-irradiation. Although most of the DNA could get repaired by the end of the 24th hour in both the PfNdr-overexpression strain and the wild-type 3D7 cell line, the enhanced early repair seen in case of PfNdr OE indicates a kinetic

advantage conferred by elevated PfNdr expression. Together, these results suggest that PfNdr plays an active role in the HR-mediated repair of DNA DSBs and that its overexpression can enhance the efficiency and speed of genome restoration following genotoxic insult in *P. falciparum*.

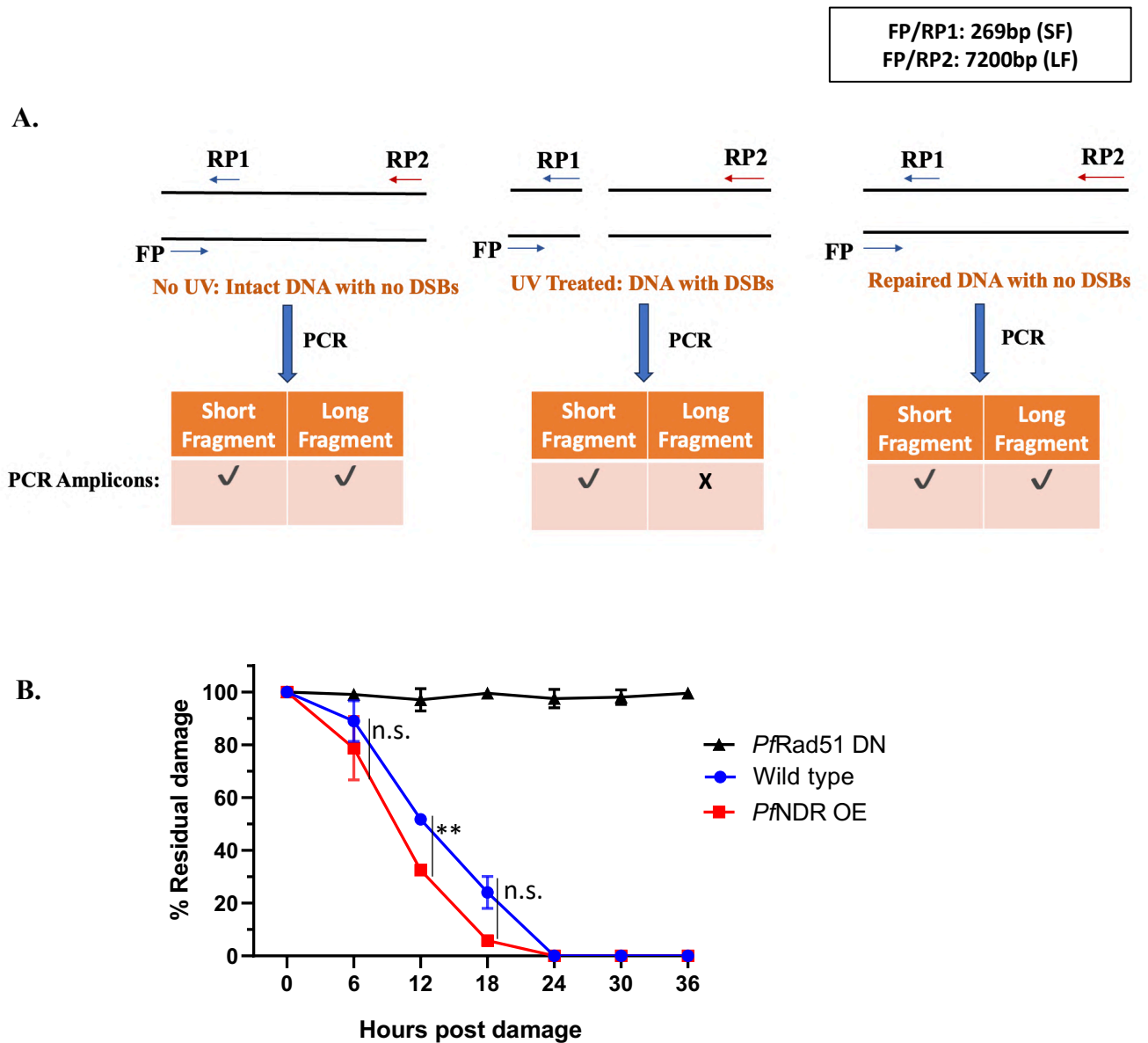


Figure 3.9. Overexpression of PfNdr provides kinetic advantage to DNA repair in malaria parasites. (A) Schematic representation of the principle of DNA DSB repair assay. The relative positions of the short- and long-range amplicons indicating the primer pairs are shown. (B) Parasite cultures were UV irradiated at the trophozoite stage. After DNA damage, cells were allowed to recover for 36 h. Repair of nuclear DNA was assayed with specific primer sets. The percent residual damage at indicated time points was plotted using GraphPad Prism. Values are mean fluorescence intensities $\pm$ SEM (error bars) of three experimental repeats. The graph displays the kinetics of nuclear DNA repair in 3D7 parasite (wild type [WT]), parasites overexpressing *PfNDR-GFP* (*PfNDR* OE) and dominant negative mutant of *PfRad51K143R* (*PfRad51 DN*) from episomal plasmids. Two-tailed Student's *t* test was used to calculate the *P* values (**, *P* value of <0.01 and N.S. means non-significant [*P* value of >0.05]).

3.6 Summary of the results:

We identified PfNdr as a novel DNA damage–responsive protein in *P. falciparum* and investigated its molecular interactions, expression dynamics, localization, and functional role in DNA repair. Bioinformatic predictions followed by yeast two-hybrid assays revealed that PfNdr interacts strongly with PfRad51 and PfDmc1, the mitotic and meiotic recombinases, respectively, and weakly with the mitochondrial polymerase PfPolG. Site-directed mutagenesis of a conserved acidic residue (D194) in PfRad51 abolished its interaction with PfNdr and other homologous recombination partners, confirming the specificity of this interaction.

Expression analysis demonstrated that PfNdr’s transcript and protein levels peak during the trophozoite and schizont stages—the mitotically active and DNA damage–prone phases of the parasite life cycle—and are co-expressed with PfRad51. Upon exposure to genotoxic stress (UV or MMS), PfNdr levels increased significantly, and immunofluorescence assays revealed its translocation from the cytoplasm to the nucleus, indicating its role in the DNA damage response.

To assess functional relevance, a PfNdr-overexpressing parasite line was compared with wild-type and PfRad51-dominant-negative mutants using a PCR-based DNA repair kinetics assay. While wild-type parasites efficiently repaired UV-induced double-strand breaks within 24 hours, PfNdr-overexpressing parasites exhibited markedly faster repair kinetics, whereas the PfRad51 mutant remained defective. Together, these findings establish PfNdr as a critical component of the homologous recombination-mediated DNA repair machinery in *P. falciparum*, whose expression and nuclear localization are induced by DNA damage and whose overexpression confers a kinetic advantage in genome restoration following genotoxic stress.

Chapter 4

Results

Deciphering the biological function of PfNdr in HR-mediated DNA repair

4.1 *PfNDR* is an orthologue of yeast RAD57 gene.

Interestingly, a BLAST analysis revealed considerable sequence similarity of PfNdr with a yeast DNA repair protein, ScRad57. It is seen that PfNdr shares a similarity of 53.17% and an identity of 19.65% with ScRad57 (Figure 4.1A). Simultaneously, PfNdr was also seen to share significant sequence similarities with other orthologs of ScRad57 in model organisms (Figure 4.1A). Our phylogenetic tree analysis also highlights the evolutionary relationship between PfNdr and Rad57/XRCC3 orthologs from model organisms (Figure 4.1B). Now in *S. cerevisiae*, ScRad57 functions as a recombination mediator and forms a stable heterodimer with ScRad55 (53,54). This Rad55–Rad57 complex together promotes the assembly and stabilization of Rad51 nucleoprotein filaments on single-stranded DNA, especially in the presence of replication protein A (RPA), which otherwise inhibits filament formation (53–56). Rad57 also helps protect Rad51 filaments from disassembly by anti-recombinase factors like Srs2, thereby facilitating homologous recombination repair of DNA double-strand breaks (57). In humans, while there is no direct Rad57 ortholog, a functional equivalent exists in the form of a Rad51 paralog, namely XRCC3 (58). The *Saccharomyces* Genome Database (SGD) also identifies XRCC3 as the human ortholog of the yeast recombination mediator protein Rad57. It is important to note that the percent identity between PfNdr and HsXRCC3 is only 14.45% which makes PfNdr a potential antimalarial drug target unlike PfRad51, which shares a significantly high identity of 61% with its human counterpart, HsRad51.

A.

	PfNdr	ScRad57	SpRhp57	DmSpnB	DrXrcc3	MmXrcc3	RnXrcc3	HsXrcc3
PfNdr	100	19.65 (53.17)	19.07 (46.24)	16.47 (46.24)	15.89 (50.57)	15.02 (45.66)	17.05 (47.10)	14.45 (45.95)
ScRad57		100	36.44 (68.64)	28.61 (63.58)	26.13 (62.78)	25.21 (63.00)	24.63 (62.02)	28.32 (63.58)
SpRhp57			100	26.39 (59.37)	28.69 (63.92)	26.64 (65.04)	26.37 (64.92)	27.16 (64.73)
DmSpnB				100	32.25 (66.56)	32.25 (63.04)	31.37 (64.80)	31.67 (64.80)
DrXrcc3					100	53.58 (82.80)	52.17 (82.60)	52.89 (84.97)
MmXrcc3						100	88.98 (96.23)	73.69 (89.88)
RnXrcc3							100	74.49 (91.30)
HsXrcc3								100

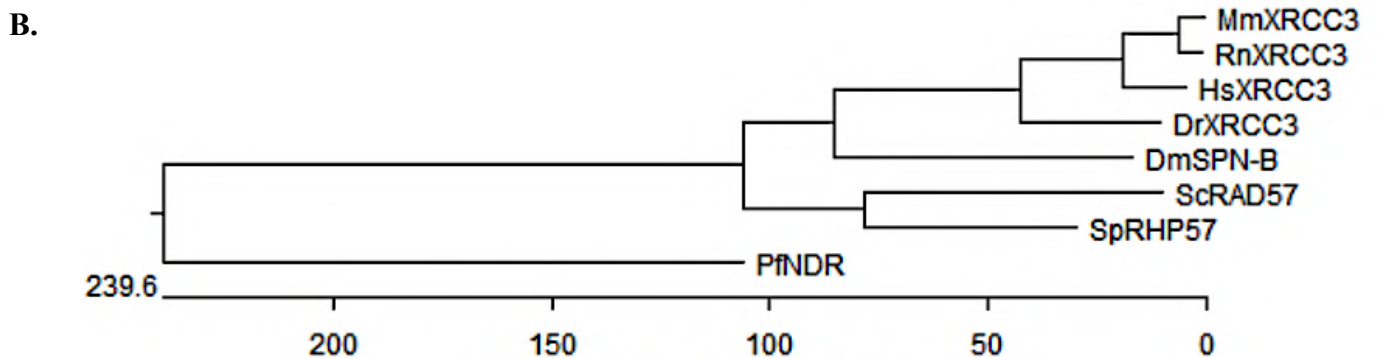


Figure 4.1. PfNdr shares similarity with Rad57 and XRCC3 orthologs from model organisms and is also phylogenetically related to them. (A) Table depicting the percent identity (in black) and percent similarity (in brackets and in orange) between PfNdr and Rad57/XRCC3 orthologs from different model organisms. (B) Phylogenetic tree depicting the evolutionary relationship between PfNdr and Rad57/XRCC3 orthologs from model organisms, made using DNA-STAR software.

4.2 PfNDR functionally complements the DNA damage sensitivity of $\Delta rad57$ mutant of *S. cerevisiae*.

In order to investigate whether PfNdr is a functional ortholog of ScRad57, we performed a yeast complementation assay. It is already known that $\Delta rad57$ mutants are defective in DNA repair (59,60). We investigated whether exogenous *PfNDR* can functionally rescue the DNA repair defect of the *ScRAD57* null mutant in budding yeast. Complementation studies were performed using an MMS spotting assay, a return-to-growth assay and a UV sensitivity assay. For our yeast complementation assay, we used a *S. cerevisiae* cell line with *ScRAD57* knockout background. *PfNDR* cloned into pRS313 vector was transformed into W303 α yeast strain. We also transformed *ScRAD57*, cloned into the pRS313 vector, and the empty pRS313 vector as positive and negative controls, respectively. Complementation studies were performed using a return-to-growth assay (Figure 4.2D) and by growing strains on plates containing 0.05% MMS (MMS spotting assay, Figure 4.2A and 4.2B). In both the assays, we observed that *PfNDR* could partially complement the DNA damage sensitivity of the $\Delta rad57$ mutant (Figure 4.2A, 4.2B and 4.2D). We also confirmed the expression of exogenous PfNdr in the $\Delta rad57$ mutant used in this study by a western blot (Figure 4.2C). Next, we exposed these three strains to three different doses of UV radiations; 50 J/m², 100 J/m², and 150 J/m². It is known that UV irradiation induces the formation of thymidine dimers in the genome (61,62). These dimers may lead to the formation of DNA DSBs when the replication fork passes through that (63). We calculated the percent survivability of each of the three strains at respective UV doses and observed that PfNdr transformed cells could reduce the UV-induced DNA damage sensitivity of $\Delta rad57$ mutant cells (Figure 4.2E). The results from these complementation studies strongly suggest that the DSB repair function of ScRad57 is conserved in PfNdr and that PfNdr is a functional ortholog of ScRad57 in *P. falciparum*.

Interestingly, as mentioned earlier, it is reported that ScRad57 functions with another yeast protein, ScRad55 in budding yeast and the physical interaction between these two proteins is

already known and established (64). When also we checked the interaction between PfNdr and ScRad55 using a yeast-two-hybrid assay, it was observed that PfNdr fails to interact with ScRad55 (Figure 4.3). This intriguing finding of a lack of interaction between PfNdr and ScRad55 in the absence of ScRad57 further validates and substantiates the partial complementation observed in our yeast complementation assay.

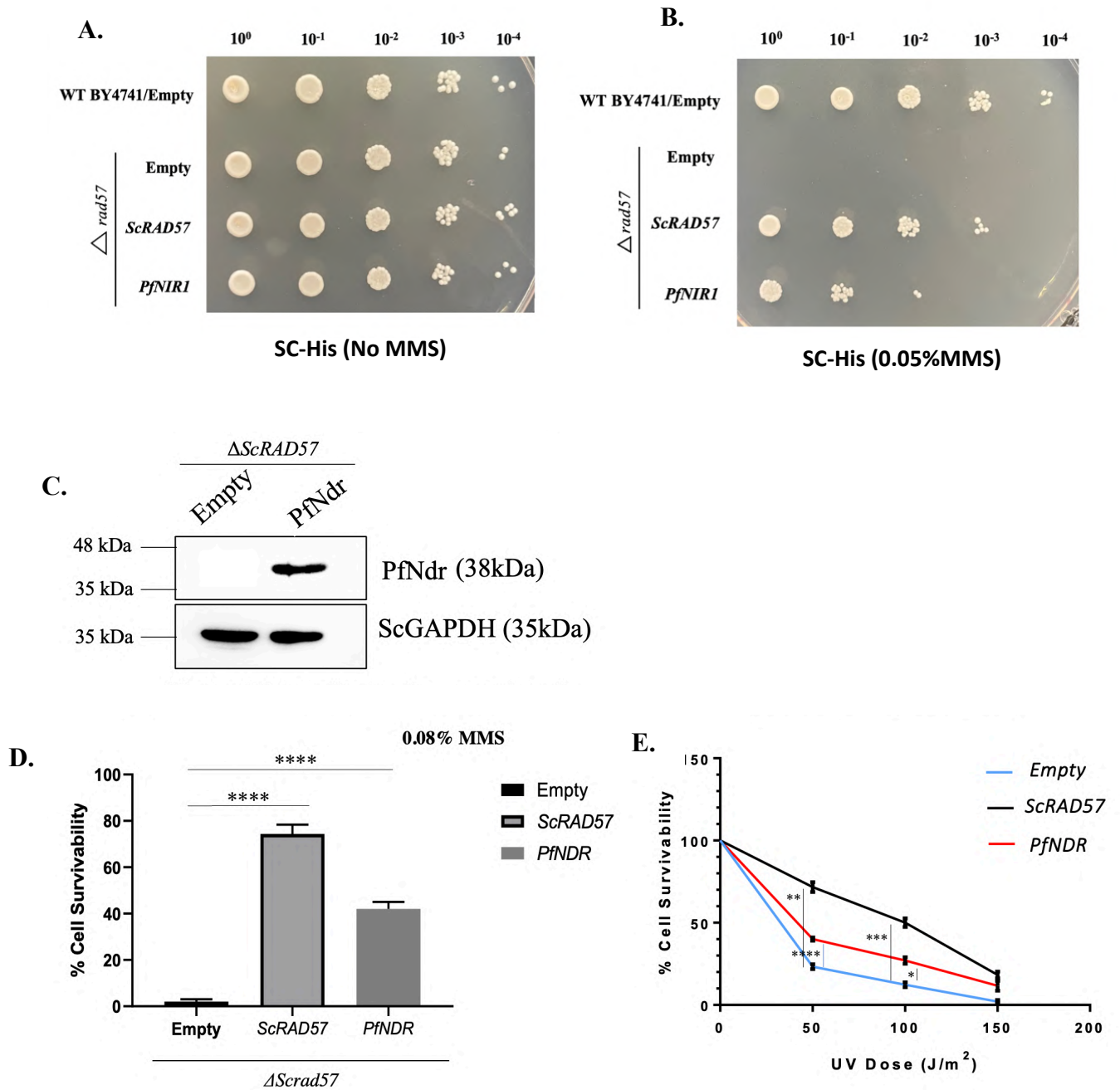


Figure 4.2. PfNdr complements the function of ScRad57. (A) MMS spotting assay in absence of MMS (B) MMS spotting assay in presence of 0.05% MMS (D) Percentage survivability of yeast strains post UV treatment with 50 J/m², 100 J/m² and 150 J/m² of UV doses. The mean value ($\pm$ SD) of three independent repeats of the above experiments was plotted; *P* values were calculated using the two-tailed Student's *t* test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) (E) Western blot demonstrating levels of PfNdr in *empty*-pRS313 and *PfNDR*-pRS313 transformed yeast strains in $\Delta rad57$ background, normalized using ScGAPDH protein. Molecular marker sizes are highlighted in the left.

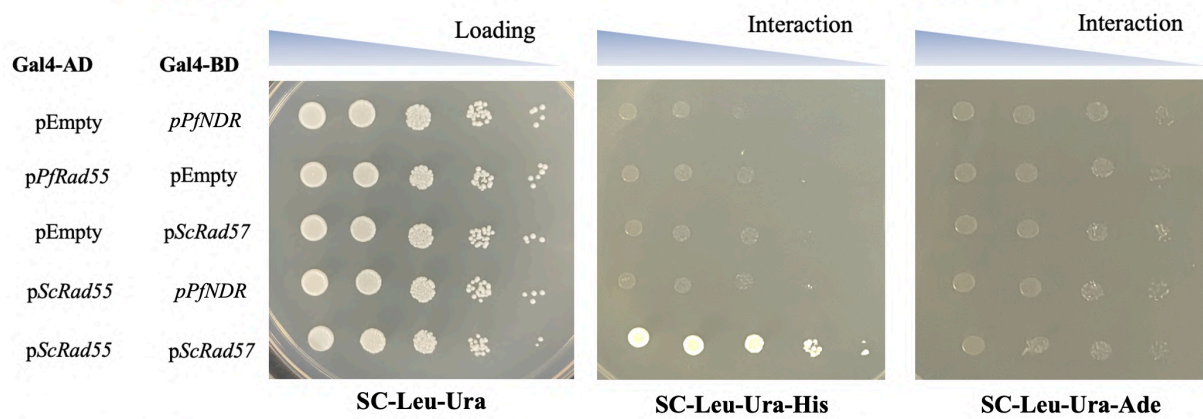


Figure 4.3. ScRad55 fails to interact with PfNdr. Yeast-two-hybrid assay that shows the lack of interaction between ScRad55 and PfNdr. Interaction between ScRad55 and ScRad57 is used as a positive control.

4.3 B02 cannot compete for the ATP binding site of PfNdr.

A previous study reports that B02 binds PfRad51 at the ATP binding site and inhibits its ATP hydrolysis and three-strand exchange activities (23). In order to check whether B02 can bind and compete with ATP for the ATP binding site of PfNdr, a molecular docking study was performed (using B02 as the ligand where B02 was docked onto PfNdr; and the binding affinities of B02 and ATP individually at the Walker A site and Dimerization Domain (DS) of PfRad51 and PfNdr respectively were calculated (Figure 4.4A and 4.4B). This bioinformatic analyses was performed in a collaboration with D. Das and A. Roy, TCS Hyderabad (Abhilasha Gahlawat *et al*, unpublished data). The binding affinities from this study suggested that B02 cannot compete with ATP and is not predicted to bind to PfNdr. However, B02 on the other hand can compete with ATP and can bind effectively to the ATP binding site on PfRad51, which is in consistent with previous results (23). Now because Rad57 is a paralog of Rad51 in *S. cerevisiae* and we know that PfNdr is a functional ortholog of ScRad57, it is highly likely that PfNdr is a paralog of PfRad51 in *P. falciparum*. PfNdr shares 17.63% identity and 48.55% similarity with PfRad51 and just like PfRad51, PfNdr also harbours the Walker A and Walker B motifs (Figure 4.4C). Docking studies performed in collaboration with Dr. Arijit Roy's lab at TCS, Hyderabad show the presence of an ATP binding pocket on PfNdr (Figure 4.4D, Abhilasha Gahlawat *et al*, unpublished data).

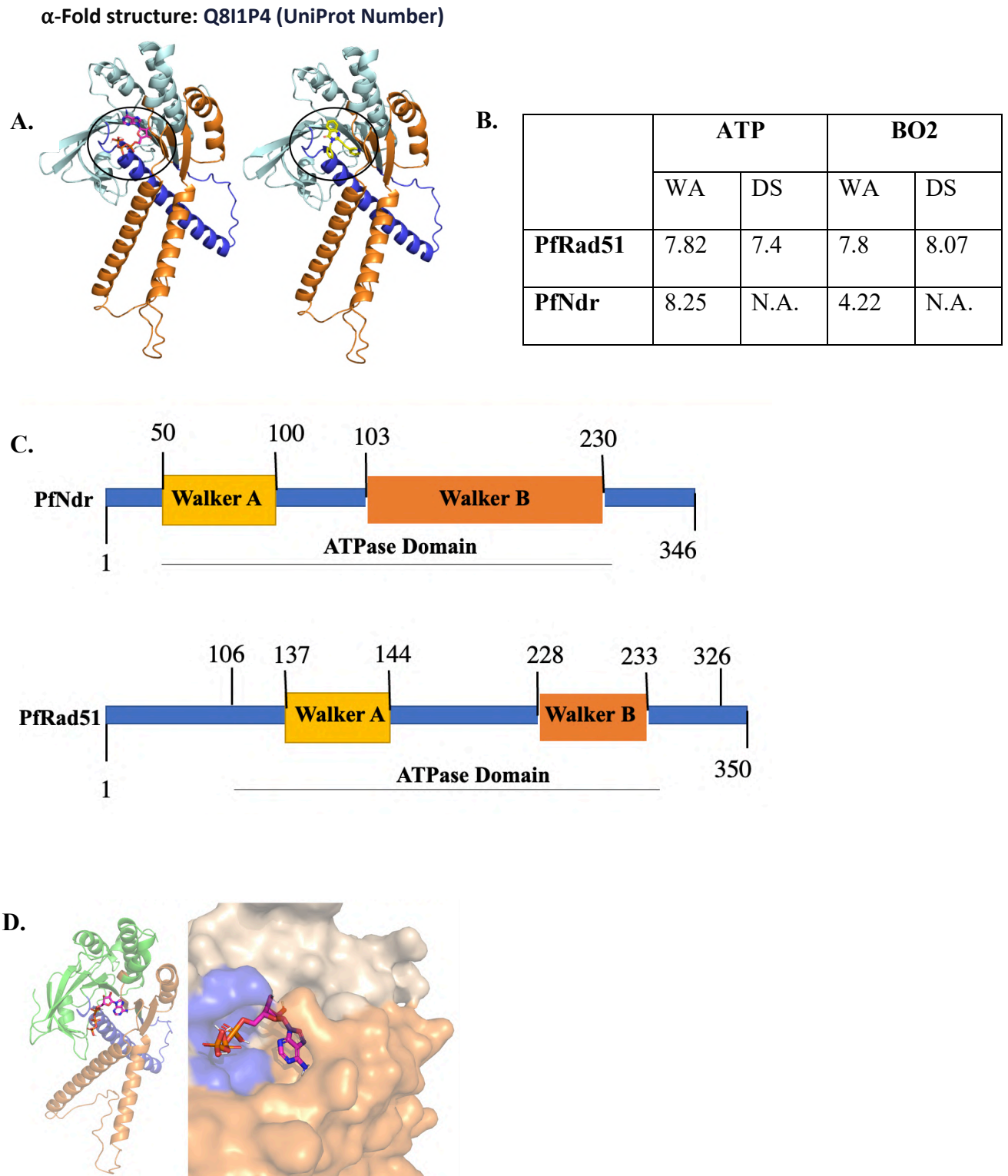


Figure 4.4. B02 cannot compete for the ATP binding site of PfNdr. (A) Molecular docking of ATP (pink sticks) and B02 (yellow sticks) docked onto PfNdr respectively. (B) Table demonstrating the binding affinities (in - kcal/mol) for ATP and B02 docked at specific sites on PfRad51 and PfNdr respectively. WA: Walker A site, DS: Dimerization Site. (C) Schematic demonstrating the conservation of Walker A motif, Walker B motif and the ATPase domain on PfNdr with respect to PfRad51. The numbers demonstrate the specific amino acid positions on each protein. (D) ATP in sticks, docked between the ATP binding site (blue) and the walker site (orange) of PfNdr.

4.4 Overexpression of PfNdr relieves the inhibition on DNA repair by PfRad51's inhibitor, B02.

Now, it is already established that in *P. falciparum*, B02 completely abrogates DSB repair by inhibiting PfRad51 (40). We hypothesize that if PfNdr is a paralog of PfRad51, overexpression of PfNdr may rescue the parasites from B02 sensitivity. To that end, we performed a similar DNA repair kinetics assay to quantify the percent residual damage in the wild-type 3D7 and PfNdr overexpression cell line, both in presence of B02 inhibitor. As expected, the wild-type 3D7 parasites failed to show any repair in presence of B02 even at the end of 36th hour post UV treatment (Figure 4.5). Wild-type 3D7 parasites in absence of B02 acted as positive control and exhibited normal DNA repair kinetics by repairing total DNA by the end of 24th hour post UV irradiation (Figure 4.5). Interestingly, overexpression of PfNdr could relieve the inhibition on DNA repair imposed by PfRad51's inhibitor, B02 (Figure 4.5). It was observed that in presence of B02, the PfNdr overexpressing parasites were able to repair 60% of its DNA by the end of 36th hour post UV irradiation unlike the wild-type parasites where the residual damage remained close to 100% even at the end of 36th hour (Figure 4.5). Hence, we conclude that PfNdr is a high copy suppressor of PfRad51 inhibition in *P. falciparum*.

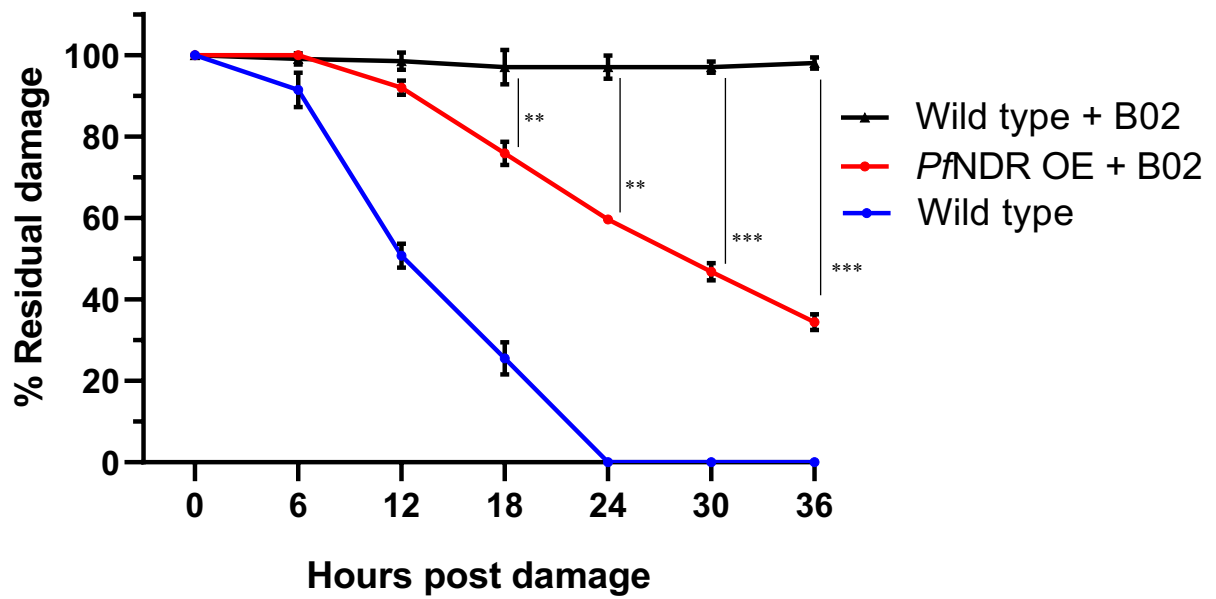


Figure 4.5. Overexpression of *PfNdr* relieves the inhibition on DNA repair by *PfRad51*'s inhibitor, B02. Parasite cultures were UV irradiated at the trophozoite stage. After DNA damage, cells were allowed to recover for 36 h. Repair of nuclear DNA was assayed with specific primer sets. The percent residual damage at indicated time points was plotted using GraphPad Prism. Values are mean fluorescence intensities $\pm$ SEM (error bars) of three experimental repeats. The graph displays the kinetics of nuclear DNA repair in 3D7 parasite (wild type [WT]) in presence and absence of B02 and for parasites overexpressing *PfNDR-GFP* (*PfNDR* OE) from episomal plasmid in presence of B02. Two-tailed Student's *t* test was used to calculate the *P* values (**, *P* value of <0.01 and N.S. means non-significant [*P* value of >0.05]).

4.5 Summary of the results:

We identified PfNdr to be putative homolog of the yeast recombination mediator protein, Rad57. Sequence alignments and phylogenetic analyses revealed ~53% similarity of PfNdr with *Saccharomyces cerevisiae* Rad57. In yeast, Rad57 functions with Rad55 to stabilize Rad51 filaments during homologous recombination repair. To assess functional conservation, we expressed *PfNDR* in a $\Delta rad57$ mutant of *S. cerevisiae* and evaluated its ability to restore DNA repair proficiency using MMS and UV sensitivity assays. PfNdr expression partially rescued the DNA damage sensitivity of the $\Delta rad57$ strain, indicating that PfNdr can complement the loss of ScRad57 and acts as its functional ortholog.

Molecular docking revealed that the Rad51 inhibitor B02 binds effectively to PfRad51 but not to PfNdr, suggesting that PfNdr lacks the B02-binding interface. Since PfNdr is a likely paralog of PfRad51, we tested whether its overexpression could relieve B02-induced inhibition of DNA repair. DNA repair kinetics assays demonstrated that PfNdr-overexpressing parasites repaired ~60% of UV-induced damage within 36 hours even in the presence of B02, whereas wild-type parasites remained unrepaired, confirming PfNdr as a high-copy suppressor of PfRad51 inhibition.

Importantly, PfNdr shares only 14.45% identity with its human ortholog XRCC3, compared to the high sequence identity between PfRad51 and HsRad51, highlighting its potential as a selective antimalarial target. Structural modelling further revealed unique, non-conserved parasite-specific regions on PfNdr that could serve as druggable surfaces for developing PfNdr-specific inhibitors.

Chapter 5

Discussion

5.1 Discussion

In this study, we report the identification and inclusive characterization of a novel DNA repair protein, PfNdr in *P. falciparum*. Our data indicates that PfNdr operates within the homologous recombination (HR) pathway, alongside the canonical recombinase PfRad51, to mediate the repair of DNA double-strand breaks (DSBs) in malaria parasites. Our bioinformatic analyses demonstrate that PfNdr is predicted to interact with other established DNA repair proteins in *P. falciparum*. Our protein-protein interaction assays demonstrate that PfNdr interacts with PfRad51 and other DNA repair proteins like PfDmc1 and PfPolG. Mechanistically, we identified a conserved Protruding Acidic Patch (PAP) on PfRad51 that is essential for its interaction with PfNdr, confirming the specificity of the interaction between them. PfNdr is seen to be maximally expressed at the DNA damage prone stages of the parasite. Furthermore, we see that PfNdr expression is significantly upregulated at both the transcript and the protein level in response to exogenous DNA damage, and it gets translocated from the cytoplasm to the nucleus under genotoxic stress conditions highlighting its functional engagement in DNA damage response mechanisms. Functionally, overexpression of PfNdr enhances the kinetics of DNA repair in the parasite and rescues the parasites from B02 sensitivity, a known small-molecule inhibitor of PfRad51. Our yeast complementation assays further support the notion that PfNdr is a functional ortholog of the yeast recombination mediator ScRad57, which stabilizes Rad51 filaments during HR in budding yeast. The data presented in this article provide compelling evidence that PfNdr is a DSB repair protein in *P. falciparum*.

A few previous studies identify the candidacy of PfRad51 to be used as an anti-malarial drug target (23,30). However, one of these study simultaneously reports the limitation associated with targeting this key recombinase, PfRad51 (23). The problem of drug toxicity due to its high homology with HsRad51 forces us to identify other key proteins of *Plasmodium* HR pathway that are less conserved in humans and that could serve as better drug targets. This study

originated from the need of identifying such less conserved DNA repair proteins in malaria parasites.

The interaction between PfNdr and PfRad51 as validated in our yeast-two-hybrid and co-purification assays, highlights the possible role of PfNdr in the same pathway as that of PfRad51. The question arises whether PfNdr functions upstream or downstream of PfRad51 in the HR pathway. The finding that PfNdr compensates for the loss of function of ScRad57 suggests us that PfNdr might play a similar role as that of ScRad57. As mentioned earlier, ScRad57 forms a heterodimer with another yeast protein, ScRad55 and this heterodimer functions together to stabilize the Rad51-nucleofilament post the resection step in the HR pathway (53–55). Thus, it is highly likely that PfNdr functions in stabilizing the PfRad51 protein onto the single-stranded DNA post the 3' to 5' resection step in the HR pathway. The ScRad57-ScRad55 heterodimer is also known to relieve the inhibition imposed on ScRad51-nucleofilament by RPA since RPA has a significantly higher binding affinity for ss-DNA as compared to ScRad51 (65,66). Such an inhibitory effect of PfNdr on RPA in *P. falciparum* should be explored in future. ScRad57 is also known to prevent the disruption of Rad51-ssDNA by the anti-recombinase, Srs2 (57). Our preliminary Y2H studies suggests that PfNdr interacts with PfSrs2 and this confirms the possible pro-recombination role of PfNdr in the HR pathway by inhibiting the translocation of PfSrs2 onto the ss-DNA. The exact collaborative mechanism of action of these repair proteins is yet to be established and should be investigated. Interestingly, no ortholog of ScRad55 is known to be present in *P. falciparum* so far. Thus, this absence of a Rad55-like protein in this protozoan parasite highlights the fact that unlike yeast and other eukaryotes, PfNdr functions by itself in malaria parasites. Our preliminary Y2H studies also suggest that PfNdr can interact with itself, showing some degree of self-interaction, demonstrating that PfNdr might have the ability to form a homo-dimer or other stable complexes by interacting with itself just like PfRad51 which forms a homo-hexamer. But the experimental validation of such a self-interaction of PfNdr must be probed into using different

assays. This will account for the absence of Rad55 in *P. falciparum*. This proposition is supported by the finding that in *S. cerevisiae*, the *Scrad55-Scrad57* double mutant exhibits a similar DNA repair defect as that of single *Scrad55* or *Scrad57* mutants suggesting that they play redundant roles (67). Interestingly, in *S. cerevisiae*, it is ScRad55 and not ScRad57 that directly interacts with ScRad51 during HR (68). There is no evidence for a direct interaction between ScRad57 and ScRad51 without ScRad55 and ScRad57's association with Rad51 is mediated through its partnership with ScRad55 in yeast (68). This further justifies the absence of Rad55 in *P. falciparum* when PfNdr is able to perform the same function.

We show that a single amino acid residue, D194 on PfRad51 is essential for its interaction with PfNdr and other auxiliary DNA repair proteins. The observation that neutralization of a single acidic amino acid (D194A) on PfRad51 disrupts critical protein–protein interactions is surprising and mechanistically significant. The Protruding Acidic Patch (PAP), a conserved region within Rad51, is fundamental to its function in recombination-mediated DNA repair (52). Negatively charged regions were identified within the corresponding α -helices of both ScRad51 and HsRad51, suggesting structural conservation of the PAP motif (52). Consistent with its evolutionary significance, sequence alignment studies demonstrate that the motif is well conserved across eukaryotes, with the key acidic residues E206 and D209 of *S. pombe* Rad57 maintained in seven of eight eukaryotic Rad51 homologs (52). Structural analyses indicate that the PAP motif is also preserved in Dmc1, the meiosis-specific member of the RecA recombinase family (52). Considering the critical role of the PAP motif's negative charge in recombinational DNA repair, it is likely that this motif engages in electrostatic interactions that help modulate Rad51 activity, potentially serving as a docking site for auxiliary factors to promote dynamic Rad51 binding.

The interaction of PfNdr with PfDmc1 observed in this study reflects the plausible roles of PfNdr in mosquito-specific sexual stages of the parasite as PfDmc1 functions in the meiotic

stages of the malaria parasite (69). Further studies on the sexual stages of the parasite can help investigating such meiotic-specific roles of PfNdr. PfPolG (or mtDNAP), the mitochondrial DNA polymerase in *P. falciparum*, plays a crucial role in the maintenance and repair of mitochondrial DNA (mtDNA) (40,70). It is also known to interact with other DNA repair proteins such as PfRad51 and PfBlm (40). A previous study establishes the recruitment of PfPolG to the parasite's mitochondrial DNA (mtDNA) and highlights the role of this nuclear encoded protein in mtDNA repair (40). The interaction of PfNdr with PfPolG provides some evidence that PfNdr might be a part of the mitochondrial recombinosome just like other nuclear encoded mitochondrial repair proteins but this needs to be further interrogated as PfNdr does not contain any mitochondrial localization signal.

Molecular docking analyses conducted in this study suggest that B02, the inhibitor of PfRad51, cannot compete with ATP for binding to PfNdr at its ATP binding site. Also, as seen in this study, overexpression of PfNdr was able to alleviate B02-mediated inhibition of homologous recombination-mediated DNA repair. This interesting finding can be rationalized by two possible models. First, in the PfNdr overexpression (OE) strain, the stoichiometric excess of PfNdr relative to PfRad51 is likely to allow even the limited fraction of unbound (and thus active) PfRad51 to be effectively stabilized on single-stranded DNA (ssDNA) by the abundant PfNdr. This stabilization may be sufficient enough to promote HR activity and partial DNA repair despite the presence of B02. Second, it is possible that B02 exhibits competitive binding affinity for both PfRad51 and PfNdr, thereby sequestering only a subset of PfRad51 and allowing residual repair activity. However, this alternative explanation is less supported, as our molecular docking studies predict that B02 fails to bind to PfNdr. Therefore, the first model is more consistent with our data, suggesting that overexpression of PfNdr mitigates the inhibitory effects of B02 by enhancing functional stabilization of the limited pool of active PfRad51 on ssDNA.

Apart from this, PfNdr is most likely an essential gene of the malaria parasite as predicted from a piggyBac transposon experiment and the data is available on PlasmoDB, where the Mutagenesis Index Score for this gene is 0.164 and Mutant Fitness Score is -2.86. Further studies can be carried out using a conditional knockout of PfNdr to elucidate its specific functions in the parasite. Most importantly, PfNdr shares only 14.45% sequence identity with its human counterpart HsXRCC3, in contrast to the 61% identity observed between PfRad51 and HsRAD51. This reduced homology minimizes the risk of host toxicity, underscoring PfNdr's potential as a parasite-specific drug target. While human XRCC3 has been implicated in both HR and DNA replication, similar multifunctional roles of PfNdr remain to be elucidated. Nevertheless, its predicted interactions with the mitochondrial polymerase, PfPolG, and DNA topoisomerases—as revealed by our Random Walk with Restart (RWR) network analysis—suggest that PfNdr may also contribute to mitochondrial DNA maintenance and other critical cellular processes. These possible diverse functions of PfNdr must be further investigated. Such multifaceted roles will collectively highlight PfNdr as a promising candidate for targeted antimalarial intervention. This study lays the foundation for future investigations aimed at evaluating the therapeutic potential and targetability of PfNdr as a novel anti-malarial drug target. Figure 5.1 here summarizes the key findings of this study. To summarize, we could identify a novel DNA repair protein, PfNdr, that:

1. Gets upregulated upon DNA damage
2. Gets translocated to the nucleus upon DNA damage
3. Is a paralog of PfRad51
4. Is a functional ortholog of ScRad57
5. Provides a kinetic advantage to the DNA DSB repair in malaria parasites
6. Is a considerable drug target to curb malaria

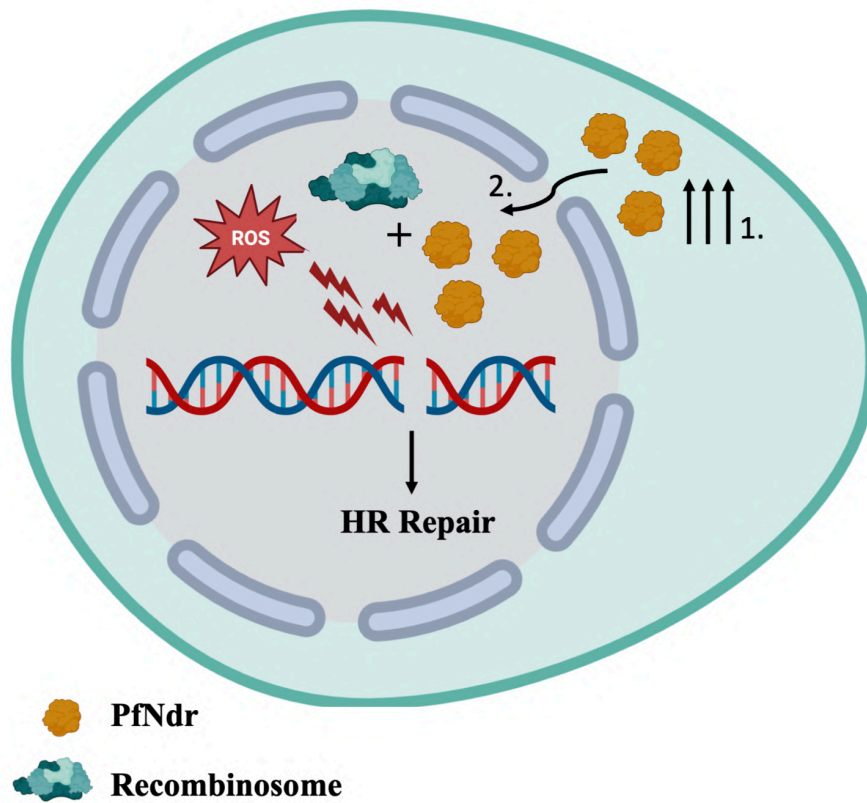


Figure 5.1. Illustration summarizing the overall role of PfNdr in parasite biology. PfNdr gets upregulated upon DNA damage and get translocated to the nucleus to repair the DNA double-strand breaks. Once within the nucleus, it functions along with other HR proteins or the recombinosome and mediates the repair of DNA DSBs for the survival of the parasite in the human host in presence of reactive oxygen species (ROS).

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Synopsis

Malaria is a serious and potentially life-threatening disease. It is a vector-borne infectious disease caused by *Plasmodium* parasites and transmitted to humans through the bite of an infected female *Anopheles* mosquito (1). In the year 2024, the World Health Organization (WHO) reported an estimated 263 million malaria cases globally, resulting in approximately 5,97,000 deaths (2). This number reflected an increase of about 11 million malaria cases as compared to the year 2022. While substantial progress has been made in reducing transmission and mortality through vector control (e.g., insecticide-treated bed nets, indoor spraying), early diagnosis, and the use of artemisinin-based combination therapies, progress has stagnated in recent years (3). Socio-environmental factors such as education, housing, and rural or migrant status significantly affect malaria awareness and prevention practices (4). Climate change has also been identified as a critical driver, with rising temperatures and precipitation levels increasing incidence globally, particularly among young children (5). New strategies including transmission-blocking vaccines and enhanced diagnostics, are being explored to overcome current challenges and meet eradication goals. Malaria is primarily found in tropical and subtropical regions and in 2023, 83 countries were identified as malaria-endemic (2). Malaria parasites have a rapid progression rate due to multiple rounds of replication within one mitotic cycle. If left untreated, it might lead to cerebral malaria, organ failure, anaemia, and other life-threatening clinical complications like respiratory distress, shock and low blood sugar. Pregnant woman and infants are more susceptible to the disease due to weaker immunities. *Plasmodium* is the genus that causes malaria, and there are five species that cause malaria in humans, namely, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. *Plasmodium falciparum* is the most dangerous malaria species and is responsible for majority of severe malaria cases and deaths.

Antimalarial drugs such as chloroquine, artemisinin, sulfadoxine-pyrimethamine, mefloquine, quinine, and atovaquone-proguanil have been central to malaria treatment, each targeting different stages of the *Plasmodium* life cycle. However, the emergence of widespread drug resistance—especially to chloroquine, and more recently to artemisinin in *P. falciparum*—has significantly undermined their clinical utility (6,7). Resistance often results from genetic mutations in parasite targets, such as *pfprt*, *pfmdr1*, and *kelch13*, which reduce drug binding or alter metabolic pathways (8,9). This escalating resistance crisis underscores the urgent need to discover and validate novel drug targets (10,11).

Plenty of DNA double-strand breaks occur naturally within the genome of malaria parasites during its asexual stages in the human host. These are generated due to errors during replication like replication fork arrest or due to the generation of free radicals produced numerous during pathways like haemoglobin detoxification or artemisinin activation. In *Plasmodium* parasites, hemoglobin digestion within the food vacuole releases free heme (ferriprotoporphyrin IX), which catalyzes the formation of reactive oxygen species (ROS), leading to oxidative stress. The parasite detoxifies this by polymerizing heme into inert hemozoin, but some free heme and ROS still persist and contribute to cellular damage. In another pathway, in presence of artemisinin, this antimalarial gets activated in *Plasmodium* parasites by interacting with free or loosely bound ferrous iron (Fe^{2+}) from digested hemoglobin. This activation cleaves artemisinin's endoperoxide bridge, generating reactive oxygen species (ROS) and carbon-centered free radicals that damage parasite proteins, membranes, and organelles. Additionally, reactive oxygen species and free radicals can cause DNA double-strand breaks (DSBs) primarily by attacking the sugar-phosphate backbone and nucleotide bases of DNA. Hydroxyl radicals, in particular, are highly reactive and can abstract hydrogen atoms from deoxyribose sugars, leading to single-strand breaks that, when closely spaced on opposite strands, result in DSBs. Additionally, oxidative modifications of bases like guanine can destabilize DNA structure and promote strand cleavage. This creates an inevitable pressure on malaria parasites to evolve coping mechanisms that are able to repair a plethora of DNA double-stranded breaks (DSBs) experienced during their asexual growth in order to survive in the human host.

Even a single unrepaired DNA DSB can lead to the death of a unicellular organism (12). The two mechanisms that repair DNA DSBs in eukaryotic organisms include Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) (13). NHEJ is completely absent in *Plasmodium* (14,15) making homologous recombination the only prominent indispensable DNA Repair machinery adopted by *Plasmodium* (16) to repair its DNA double-strand breaks. Homologous recombination (HR) is a high-fidelity DNA repair mechanism that resolves double-strand breaks (DSBs) by using a homologous DNA sequence—typically the sister chromatid—as a template for accurate repair. The process begins with the recognition of the DSB, followed by end resection to generate 3' single-stranded DNA overhangs. These overhangs are coated with Rad51, which facilitates strand invasion into the homologous duplex DNA. DNA synthesis extends the invaded strand using the intact template, and the resulting joint molecule is resolved to restore the DNA's original structure without loss of genetic information. HR is essential for maintaining genome integrity during replication and after DNA

damage from sources like ionizing radiation or reactive oxygen species. This mechanism plays a crucial role in both normal cell function and in the survival of pathogens like *P. falciparum*, which rely on HR to repair damage caused by oxidative stress and antimalarial drugs.

Rad51, the homologous recombination recombinase in *P. falciparum* (PfRad51), is a central player in the repair of DNA double-strand breaks (DSBs), which are as mentioned earlier, frequent during replication and oxidative stress. PfRad51 catalyzes ATP-dependent strand exchange and is upregulated in response to DNA damage, indicating its active role in maintaining genome stability (17). Mechanistically, PfRad51 interacts with accessory proteins like Rad54 and replication protein A (PfRPA) (18). Rad51 has been recently studied as a potential antimalarial drug target where a small-molecule inhibitor, B02, has been shown to disrupt its strand-exchange and ATPase activities, sensitizing parasites to artemisinin and chloroquine (16). Moreover, inhibition of PfRad51 combined with Hsp90 blockers leads to severe DNA damage and reduced parasite viability, emphasizing its druggable potential (19). However, there is a research gap here and targeting PfRad51 as an antimalarial drug target poses a limitation as the ortholog of PfRad51 in humans, HsRad51, shares a significant identity of 61% with PfRad51. And, we know that, targeting *Plasmodium* repair proteins that have high sequence identity with their human orthologs may impart drug toxicity to humans as well. Having said that, identifying novel *Plasmodium* specific proteins or proteins which share less homology with their human counterparts and those that are essential for the HR-mediated DNA repair in the parasite, can serve as promising therapeutic targets for antimalarial drug development due to its essential role in DNA repair and parasite survival.

In a quest to identify such a drug target, a Random Walk with Restart (RWR) analysis revealed a novel DNA repair candidate in *P. falciparum* and we named this protein as PfNdr (Novel DNA Repair protein). A protein–protein interaction (PPI) network was constructed using known DNA repair genes as seed nodes and a previously uncharacterized gene, PF3D7_0419300, emerged as a top-ranking candidate within the network, while most other high-scoring genes were already characterized or corresponded to previously annotated DNA repair factors. PfNdr (PF3D7_0419300) is predicted to functionally associate with the DNA repair machinery in *P. falciparum* (D. Das and A. Roy: unpublished data). We performed a multiple sequence alignment (MSA) by taking the sequence of PfNdr and its orthologs from other *Plasmodium* species. Our phylogenetic analysis revealed that PfNdr is evolutionarily conserved across multiple *Plasmodium* species, suggesting a functionally important and

possibly conserved role in parasite biology. Hence, from this preliminary data, we hypothesize that PfNdr might have a role in the homologous recombination mediated DNA DSB repair of malaria parasites. To this end, we performed some experiments and validated the following results.

A yeast-two-hybrid assay was employed that showed that PfNdr interacts with other known DNA repair proteins like the mitotic recombinase, PfRad51; the meiotic recombinase, PfDmc1; and the mitochondrial polymerase, PfPolG. Parallely, a study arising from structural and biochemical analyses of Rad51 in *S. pombe*, identified an evolutionarily conserved acidic patch on Rad51—formed by residues E205, E206, and D209—that is essential for recruiting key auxiliary proteins (Rad55–Rad57, Rad52, Rad54) and enabling efficient homologous recombination repair of DNA double-strand breaks (20). This acidic motif is being referred to as PAP (Protruding Acidic Patch) and it was reported that neutralizing this motif disrupted these interactions of Rad51 with its auxiliary repair proteins, completely abolishing Rad51-mediated repair *in vivo*. We then performed a multiple sequence alignment to establish the presence of such an acidic motif on PfRad51 and we found that the PAP motif on PfRad51 was composed of a single acidic amino acid *i.e.*, D194. We mutated this aspartic acid to a neutral alanine using Side Directed Mutagenesis (SDM) and checked the interaction of PfRad51 with other DNA repair proteins using the yeast-two-hybrid assay. As expected PfRad51^{D194A} failed to interact with other DNA repair proteins like PfNdr, PfTopo3, PfBlm and PfPolG. Wildtype PfRad51, on the other hand, was able to interact with these proteins as seen in the yeast-two-hybrid assay. We also validated the protein expression of both, the PAP mutated PfRad51^{D194A} and the WT PfRad51 using a western blot analysis with -PfRad51 antibody that confirmed the presence of these transformed proteins in the yeast strains used in our assay. To further ascertain this, we resorted to perform an alternate *in vitro* co-purification assay where we checked the interaction between PfRad51 and PfNdr using a Ni-NTA affinity column. The presence of PfNdr in the bound protein lysate fraction in this assay confirmed the specificity of interaction between PfRad51 and PfNdr.

We examined the stage-specific expression profile of PfNdr at both the transcript and protein levels across the three intra-erythrocytic stages of *P. falciparum*—ring, trophozoite, and schizont. Semi-quantitative real-time PCR revealed that PfNdr transcript levels peaked during the trophozoite and schizont stages, which correspond to the S-phase and are characterized by active mitosis and elevated DNA replication stress. Notably, PfNdr expression was seen to be

congruent to that of PfRad51, a known homologous recombination key protein, at both transcript and protein levels, with maximal expression at the mitotic stages. To investigate their response to genotoxic stress and DNA damage, parasites were subjected to UV irradiation. A dose-dependent upregulation of PfNdr and PfRad51 transcripts was observed, indicating a potential role of PfNdr in the DNA damage response pathway. Furthermore, following a sub-lethal UV dose (100 J/m²), stage-specific analysis showed maximal induction at the trophozoite and schizont stages, with ~7-fold and ~10-fold transcript increases for PfNdr and PfRad51, respectively. These findings suggest that PfNdr is a DNA damage-responsive gene co-regulated with PfRad51 and likely functions in homologous recombination during the parasite's mitotically active stages.

In order to investigate the sub-cellular localization of PfNdr under both normal conditions and under DNA-damaging conditions, we performed an immunofluorescence assay using DAPI as a nuclear marker. Fluorescence intensity of PfNdr was quantified in the cytoplasm and nucleus using ImageJ in untreated parasites and in parasites treated with 0.05% MMS (a DNA-damaging agent). In untreated cells, PfNdr was seen to be predominantly cytoplasmic whereas MMS-treated parasites showed a marked nuclear accumulation of PfNdr. Quantitative colocalization analysis using Pearson's Correlation Coefficient (PCC) revealed a significant increase in nuclear overlap—from 0.279 in untreated cells to 0.663 following MMS treatment—indicating translocation of PfNdr to the nucleus in response to DNA damage. These findings suggest that PfNdr dynamically localizes to the nucleus under genotoxic stress, potentially participating in nuclear DNA repair pathways.

To assess the impact of PfNdr on DNA repair kinetics, we quantified the double-strand break (DSB) repair in three *P. falciparum* strains: wild-type 3D7, a PfRad51 dominant negative (DN) mutant (PfRad51^{K193R}), and a PfNdr overexpression (OE) line generated in this study. The dominant negative mutant inhibits the wild-type PfRad51 function despite the presence of wild-type protein, as established in prior studies (14). The PfNdr overexpression strain was generated in our study using an overexpression plasmid and expresses an exogenously overexpressing GFP-tagged PfNdr. We employed a previously described PCR-based DSB detection method to monitor percent repair or residual DNA damage over time (21). This method is based on the principle that a longer region of the genome is likely to experience more UV-induced DNA breaks and as a result, will not yield a PCR amplicon until and unless such DNA breaks are repaired. On the other hand, a short region of the genome could be amplified

even under DNA-damaging conditions as the likelihood of occurrence of a DNA DSB in a short stretch of the genome is less as compared to the longer fragment. Thus, this assay relies on the differential amplifiability of long vs short genomic regions—where long regions fail to amplify if DSBs are present. Parasites were harvested at 0, 6, 12, 18-, 24-, 30-, and 36-hours post-UV irradiation, and percent residual DNA damage was calculated. In the PfRad51 DN strain, residual damage remained near 100% throughout, indicating a complete block in DSB repair. In contrast, the wild-type 3D7 line showed progressive repair, with most DNA damage resolved by 24 hours, consistent with previous findings (22). Interestingly, the PfNdr OE line exhibited an accelerated repair phenotype, showing a clear reduction in residual DNA damage by 12 hours, suggesting a kinetic advantage in DNA repair. These results support a role for PfNdr in facilitating timely DSB repair in *P. falciparum*.

Interestingly, a BLAST analysis revealed considerable sequence similarity of PfNdr with a yeast DNA repair protein, ScRad57. It is seen that PfNdr shares a similarity of 53.17% and an identity of 19.65% with ScRad57. Our protein of interest, PfNdr, was also seen to share significant sequence similarities with other orthologs of ScRad57 in model organisms. Our phylogenetic tree analysis also further highlights the evolutionary relationship between PfNdr and Rad57/XRCC3 orthologs from model organisms. Now in *Saccharomyces cerevisiae*, Rad57 functions as a recombination mediator and forms a stable heterodimer with Rad55 (23). This Rad55–Rad57 complex together promotes the assembly and stabilization of Rad51 nucleoprotein filaments on single-stranded DNA, especially in the presence of replication protein A (RPA), which otherwise inhibits filament formation (23). Rad57 also helps protect Rad51 filaments from disassembly by anti-recombinase factors like Srs2, thereby facilitating homologous recombination repair of DNA double-strand breaks (23). In humans, while there is no direct Rad57 ortholog, a functional equivalent exists in the form of a Rad51 paralog, namely XRCC3. The *Saccharomyces* Genome Database (SGD) also identifies XRCC3 as the human ortholog of the yeast recombination mediator protein Rad57. It is important to note that the percent identity between PfNdr and HsXRCC3 is only 14.45% which makes PfNdr a potential antimalarial drug target unlike PfRad51, which shares a significantly high identity of 61% with its human counterpart, HsRad51. In order to further validate if PfNdr is a functional ortholog of ScRad57, we performed a yeast complementation assay. We investigated whether exogenous *PfNDR* can functionally rescue the DNA repair defect of the *ScRAD57* null mutant of budding yeast. Complementation studies were performed using an MMS spotting assay, a return-to-growth assay and a UV sensitivity assay. In all the three assays, we observed that

PfNDR could partially complement the DNA damage sensitivity of the $\Delta rad57$ mutant. This suggests that PfNdr is a functional ortholog of the yeast DNA repair protein, ScRad57. Interestingly, as mentioned earlier, it is reported that ScRad57 functions with another yeast protein, ScRad55 in budding yeast and the physical interaction between these two proteins is already known and established (24). When we checked the interaction between PfNdr and ScRad55 using a yeast-two-hybrid assay, it was seen that PfNdr fails to interact with ScRad55. This intriguing finding of a lack of interaction between PfNdr and ScRad55 in the absence of ScRad57 further validates and substantiates the partial complementation observed in our yeast complementation assay.

In addition to this, PfNdr shares 17.63% identity and 48.55% similarity with PfRad51. Like PfRad51, PfNdr harbors the Walker A and Walker B motifs and the ATPase domain. A docking study performed using ATP as the ligand onto the α -fold structure of PfNdr (UniProt Number: Q8I1P4) confirms the presence of an ATP binding site on PfNdr. Interestingly, Rad55 and Rad57 are paralogs of Rad51 in yeast and considering the fact that PfNdr is an ortholog of ScRad57, these findings allow us to speculate if PfNdr is a paralog of PfRad51 in *P. falciparum*. Furthermore, a previous study from the lab reported that B02, a small-molecule inhibitor of PfRad51, binds PfRad51 at the ATP binding site and inhibits its ATP hydrolysis and three-strand exchange activities (16). We then wanted to check if B02 could bind and compete with the ATP binding site of PfNdr. To that end, we performed a docking study using B02 as the ligand onto PfNdr and calculated the binding affinities of B02 vs ATP at the Walker A and Dimerization Domain (DS) of PfRad51 and PfNdr. Our results suggest that B02 cannot compete with ATP and is not predicted to bind to PfNdr. B02, on the other hand can compete with ATP and can bind effectively to the ATP binding site on PfRad51, which is in consistent with previous results (16). Importantly, it is already established that in *Plasmodium*, the PfRad51 inhibitor B02, completely abrogates DNA DSB repair (22). Hence, we hypothesize that if PfNdr is a paralog of PfRad51, overexpression of PfNdr may rescue the parasites from B02 sensitivity. In order to validate that, we quantified the percent residual damage of the wild-type 3D7 cell line and PfNdr overexpressing cell line post DNA damage in presence of B02. Wildtype parasites, in consistent with our previous findings, failed to show any repair in presence of B02. However, overexpression of PfNdr could relieve the inhibition on DNA DSB repair of the parasites imposed by B02. Thus, we can conclude that PfNdr is a high copy suppressor of PfRad51 inhibition in *P. falciparum*.

To summarize, this study could identify and characterize a novel DNA repair protein, PfNdr, in *P. falciparum*. We find that PfNdr belongs to the same pathway as that of PfRad51, *i.e.*, homologous recombination (HR) and it repairs DNA DSBs using the HR-mediated DNA repair pathway. We validate that PfNdr interacts with PfRad51 and other DNA repair proteins; gets upregulated upon exogenous DNA damage both, at the transcript and the protein level and gets translocated to the nucleus under exogenous DNA stress. We also confirm the presence of a Protruding Acidic Patch (PAP) motif on PfRad51 that is crucial for its interaction with PfNdr. We validate that overexpression of PfNdr provides kinetic advantage to the DNA repair of malaria parasites and rescues them from B02 sensitivity. On the basis of our yeast complementation studies, we propose PfNdr to be a functional ortholog of a yeast DNA repair protein, ScRad57. We posit that PfNdr could serve as a potential novel anti-malarial drug candidate. This is based on the fact that PfNdr shares a minimal sequence identity of only 14.45% with its human counterpart, HsXRCC3, unlike PfRad51 which shares 61% sequence identity with HsRad51 and thus limits the problem of drug toxicity in humans. HsXRCC3 in humans has also been reported to be involved in DNA replication but such functions of PfNdr are yet to be explored. However, its interaction with PfPolG and other topoisomerases, as seen in our RWR analysis, highlights the fact that it is likely to be involved in mitochondrial DNA repair and in other critical pathways as well. These probable multifaceted roles of PfNdr further supports its potential as a druggable target for malaria intervention.

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Identification and Functional Characterization of a Novel Component of the DNA Repair Machinery of Malaria Parasites

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Hsp90 and associates shaping parasite biology

Abhilasha Gahlawat,¹ Sunanda Bhattacharyya,² Mrinal Kanti Bhattacharyya¹

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ABSTRACT Hsp90 is considered to be the master regulator of chaperone activity within the cellular context. In addition to aiding client maturation and maintaining protein homeostasis, Hsp90 serves various non-canonical functions in model eukaryotes: ranging from protein-trafficking into the nucleus to transcriptional regulation, from chromatin remodeling to assembly and disassembly of protein complexes during DNA repair and telomere maintenance. In performing all these trades, Hsp90 collaborates with its co-chaperones in a client-specific or function-specific manner. Hsp90 undergoes various conformational changes during its chaperone cycle, which is regulated via several post-translational modifications (PTM). Different combinations of such PTMs, known as the chaperone code, also play key regulatory roles for Hsp90 functions. Here, we examine various cellular functions of Hsp90 in protozoan parasites, particularly those that shuttle between insect host and human host, adapting to a temperature difference of at least 10°C. Our analyses reveal that most of the prominent co-chaperones are present in all these parasites, except for one that is essential in model eukaryotes. We reviewed the biochemical correlates of Hsp90 and its co-chaperone interactions and realized that the physiological significance of such interplay is largely unknown in the protozoan parasites. One striking observation is the lack of sequence conservation of the parasitic co-chaperones with their human counterparts, making them attractive drug targets. Our analyses revealed that in spite of the identification of few PTMs of parasitic Hsp90 proteins, the chaperone codes remain largely elusive.

IMPORTANCE Hsp90 is a pivotal molecular chaperone involved in maintaining proteostasis and facilitating the maturation of diverse client proteins. Beyond its canonical folding functions, Hsp90 plays non-canonical roles in nuclear trafficking, transcriptional regulation, chromatin remodeling, and DNA repair. These activities are tightly regulated through interactions with specific co-chaperones and through post-translational modifications, collectively forming the “chaperone code.” This study examines Hsp90’s role in thermal adaptation of protozoan parasites when shuttling between the insect and human hosts. Here, we summarize the canonical and diverse non-canonical functions of Hsp90 in three protozoan parasites: *Plasmodium*, *Leishmania*, and *Trypanosoma*. We highlight all the Hsp90 isoforms found in these three parasites and also illustrate all the co-chaperones and post-translational modifications of Hsp90 found to be present in these protozoan parasites. Importantly, the divergence in co-chaperone sequences from human homologs in these parasites presents a promising avenue for targeted antiparasitic drug discovery and development.

KEYWORDS Hsp90, co-chaperone, chaperone code, *Plasmodium*, *Trypanosoma*, *Leishmania*

Protozoan parasites *Plasmodium*, *Leishmania*, and *Trypanosoma* collectively affect one-fourth of the global population despite encountering a plethora of challenges in the host environment. A major challenge for these parasites is adapting to temperature fluctuations as they transit from the insect vectors to human hosts. *Plasmodium*

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Address correspondence to Mrinal Kanti Bhattacharyya, mkbsl@uohyd.ac.in.

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Bloom Helicase Along with Recombinase Rad51 Repairs the Mitochondrial Genome of the Malaria Parasite

Payal Jha^a, Abhilasha Gahlawat^a, Sunanda Bhattacharyya^b, Sandeep Dey^c, Kota Arun Kumar^c, Mrinal Kanti Bhattacharyya^a

^aDepartment of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, India

^bDepartment of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, India

^cDepartment of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad, India

ABSTRACT The homologous recombination (HR) pathway has been implicated as the predominant mechanism for the repair of chromosomal DNA double-strand breaks (DSBs) of the malarial parasite. Although the extrachromosomal mitochondrial genome of this parasite experiences a greater number of DSBs due to its close proximity to the electron transport chain, nothing is known about the proteins involved in the repair of the mitochondrial genome. We investigated the involvement of nucleus-encoded HR proteins in the repair of the mitochondrial genome, as this genome does not code for any DNA repair proteins. Here, we provide evidence that the nucleus-encoded “recombinosome” of the parasite is also involved in mitochondrial genome repair. First, two crucial HR proteins, namely, *Plasmodium falciparum* Rad51 (PfRad51) and *P. falciparum* Bloom helicase (PfBlm) are located in the mitochondria. They are recruited to the mitochondrial genome at the schizont stage, a stage that is prone to DSBs due to exposure to various endogenous and physiologic DNA-damaging agents. Second, the recruitment of these two proteins to the damaged mitochondrial genome coincides with the DNA repair kinetics. Moreover, both the proteins exit the mitochondrial DNA (mtDNA) once the genome is repaired. Most importantly, the specific chemical inhibitors of PfRad51 and PfBlm block the repair of UV-induced DSBs of the mitochondrial genome. Additionally, overexpression of these two proteins resulted in a kinetically faster repair. Given the essentiality of the mitochondrial genome, blocking its repair by inhibiting the HR pathway could offer a novel strategy for curbing malaria.

IMPORTANCE The impact of malaria on global public health and the world economy continues to surge despite decades of vaccine research and drug development efforts. An alarming rise in resistance toward all the commercially available antimalarial drugs and the lack of an effective malaria vaccine brings us to the urge to identify novel intervention strategies for curbing malaria. Here, we uncover the molecular mechanism behind the repair of the most deleterious form of DNA lesions on the parasitic mitochondrial genome. Given that the single-copy mitochondrion is an indispensable organelle of the malaria parasite, we propose that targeting the mitochondrial DNA repair pathways should be exploited as a potential malaria control strategy. The establishment of the parasitic homologous recombination machinery as the predominant repair mechanism of the mitochondrial DNA double-strand breaks underscores the importance of this pathway as a novel druggable target.

KEYWORDS PfBlm, PfRad51, mitochondria, DNA repair, homologous recombination, *Plasmodium falciparum*

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