Functional characterization of *Plasmodium falciparum* DNA repair protein PfalMre11

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Department of Biochemistry

BY

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DECLARATION

I, **B. Sugith Babu** hereby declare this thesis entitled, **Functional characterization *Plasmodium falciparum* DNA repair protein PfalMre11** submitted by me, under the supervision of **Dr. Mrinal Kanti Bahattacharyya**, 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 institute for the award of any degree or diploma.

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DECLARATION

I, **B. Sugith Babu** hereby declare that this thesis entitled **"Functional Characterization of *Plasmodium falciparum* DNA Repair Protein PfalMre11"**. Submitted by me under the guidance and supervision **Dr. Mrinal Kanti Bhattacharyya, Associate Professor, Department of Biochemistry, University of Hyderabad** is a bonafide research work which is also free from plagiarism. 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 Shodganga/INFLIBNET.

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CERTIFICATE

This is to certify that this thesis entitled **"Functional characterization of *Plasmodium falciparum* DNA repair protein PfalMre11"** is a record of bona fide work done by **B. Sugith Babu**, a research scholar for PhD programme in Department of Biochemistry, School of Life sciences, University of Hyderabad under my guidance and supervision. This thesis has not been submitted previously in part or in full to this or any University or Institute for the award of any degree or diploma.

Dr. Mrinal Kanti Bhattacharyya

Head of the Department **Principal Investigator**

Dean of the School

Dedicated to my beloved
family members and
my teacher
Late Dr. Jyotsna Kumari

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ABBREVIATIONS

aa	Amino acid
Ade	Adenine
Amp	Ampere
A-NHEJ	Alternate Non-homologous end joining
ARP	Asparagine rich protein
ATR	Ataxia-telengiactasia mutated and Rad3-related
BIR	Break Induced Repair
bp	Base pair
$CaCl_2$	Calcium Chloride
cDNA	Complementary DNA
C-NHEJ	Classical Non-homologous end joining
CPDA-1	Citrate Phosfate Dextrose Acetate-1
Da	Dalton
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA protein kinase catalytic domain
DSB	Double strand break
dsDNA	double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
Htc	Hematocrit
HR	Homologous recombination
IPTG	Isopropyl thiogalactosidase
iRBC	infectefed RBC
KCl	Potassium Chloride

kDa	Kilo Dalton
LB	Luria-bertani broth
Leu	Leucine
LiOAC	Lithium Acitate
ml	Milli liter
MMS	Methyl methane sulfonate
MRE11	Meiotic recombination 11
MRX	Mre11-Rad50-XRS2
Mwt	Molecular weight
NaCl	Sodium Chloride
NaOAc	Sodium Acitate
NHEJ	Non homologous end joining
OD	Optical Density
ORF	Open reading frame
PARP-1	Poly ADP ribose polymerase-1
PBS	Phosphate buffered saline
PCIA	Phenyl Chloroform Isoamyl alcohol
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PVDF	Poly vinylidene Fluoride
Rad50	Radiation sensitive 50
Rad51	Radiation sensitive 51
RBC	Red Blood Cell
RNA	Ribonucleic acid
RNase	Ribonuclease

RPA	Replication protein A
rpm	rotation per minute
RT	room temperature
SC	synthetic complete
SDS	sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate Poly acryl amide gel electrophoresis
SSA	Single strand annealing
ssDNA	Single strand DNA
TBE	Tris Borate EDTA
TCA	Trichloroacetic acid
TE	Tris EDTA
Tric Cl	Tris Chloride
TRP	Tryptophan
Ura	Uracil
Wt	Wild type
XRS2	X-ray sensitive 2
YPD	Yeast extract dextrose
µg	Microgram
µl	Microliter

INTRODUCTION

1.1. Malaria:

Malaria is a life-threatening infectious disease caused by apicomplexan protozoan parasites *Plasmodium*. In 1897 Sir Ronald Ross discovered that malaria parasites are transmitted by female Anopheles mosquito. About 200 species of *Plasmodium* parasites cause malaria in animals. Among them five different species were found to cause malaria in humans. They are *P. falciparum, P. vivax, P.malariae, P. ovale* and *P. knowlesi*. Infection with *P. falciparum* causes severe malaria due to long period of paroxysm. It causes many complications in pregnancy and is responsible for cerebral malaria and severe anemia. Malaria prevalence is majorly observed in tropical, sub-tropical areas. WHO (World Health Organization) has reported that world's malaria prevalence is very high in African countries: accounting for majority of deaths due to malaria infection. WHO has also reported that 219 million malaria cases have been documented in 2010 to 2013, and the mortality rate was approximately 1 million people, of which most of the deaths were found in children under the age group of 5.

1.1.1 Life cycle:

The *Plasmodium* parasites have complex life cycle. They depend on two hosts to complete their life cycle. One of the hosts is mosquito which spreads malaria while taking up blood meal from a person suffering from malaria. Within mosquito midgut, parasites differentiate into male and female gamete and develop into a motile form (ookinete) which migrates through the midgut epidermal layer and are transformed into oocyst. Oocyst multiplies and releases thousands of sporozoites that reside in the salivary glands of the mosquito. When mosquito bites human for blood meal, the parasites get transmitted to human in the form of sporozoites. Sporozoites invade hepatocytes by traveling through the bloodstream and then propagate to produce merozoites which initiate asexual life cycle of the parasite. Merozoites invade erythrocyte and propagate through three developmental stages, namely ring, trophozoite and schizont stage. The

schizont eventually releases merozoites through rupturing the infected erythrocytes and infect new erythrocytes (Figure 1) (Derbyshire et al. 2011).

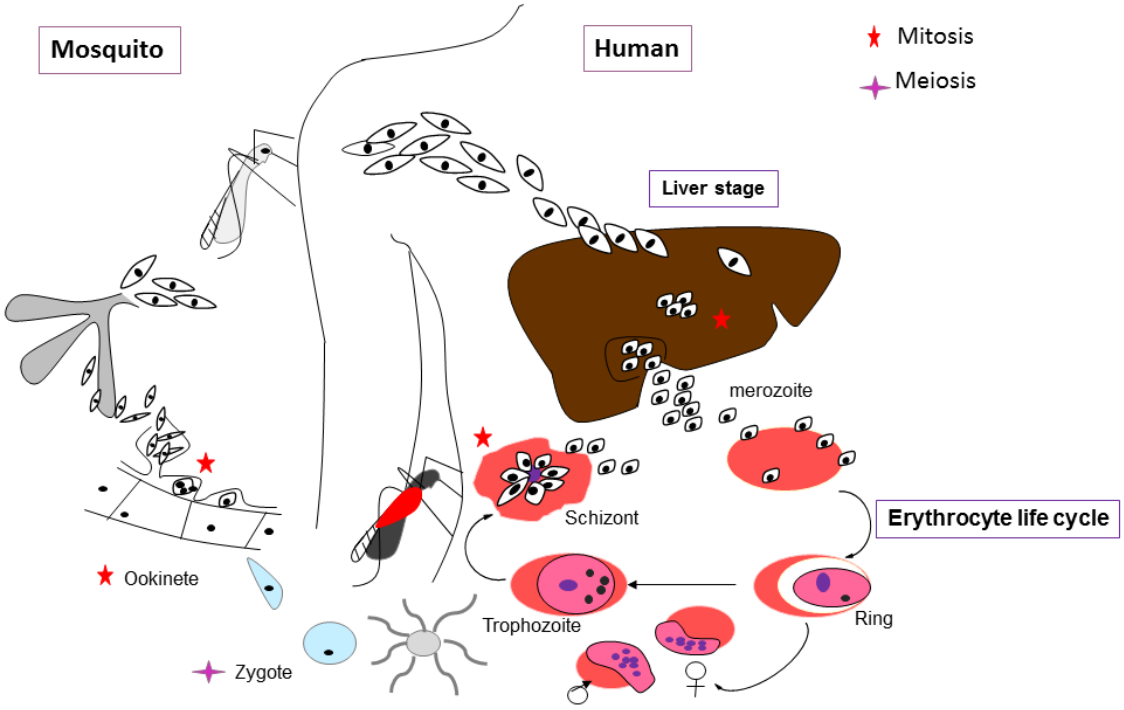


Figure 1: Schematic diagram of *Plasmodium falciparum* life cycle (adopted from Cowman et al. 2012. J. Cell Bio).

1.1.2. Drug against malaria:

The first successful and safest drug used to treat malaria was chloroquine (CQ). Later many anti-malarial drugs such as mefloquine, proguanil, primaquine, and atovaquone were also introduced under malaria eradication campaign program. *P. falciparum* developed resistance to chloroquine and its derivatives in many endemic areas. Thereafter, Artemisinin has emerged as a promising drug against malaria. While most of the drugs are active against blood stages of the parasite, a very few drugs like anti-folates, atovaquone are effective against the liver stage of parasites. Primaquine effectively eradicates liver and blood stages of the parasite; however it shows side effects under glucose-6- dehydrogenase deficient condition (reviewed in Flannery et al. 2013).

Malaria treatment has become difficult as the parasites developed resistance to most of the available drugs. Chloroquine served as an excellent drug to eradicate malaria till 1970's. Thereafter parasite developed resistance against that drug. Artemisinin administration could control malaria and it is used in combination therapy along with other drugs (Aguiar et al. 2012) which is known as Artemisinin Combination Therapy (ACT). Artemisinin generates reactive oxygen species (ROS) which leads to severe DNA damage in the parasite (Gopalakrishnan et al. 2015), also artemisinin specifically inhibits the PfATP6 activity in Oocysts (Eckstein-Ludwig et al. 2003). However, there were reports that malaria parasites have developed resistance against artemisinin in Thai-Cambodian border (Dondorp et al. 2010). Thus, there is an urgent need to find the new drug targets to treat malaria. The first thing would be to identify weak points of the parasites in various metabolic pathways as potential drug targets. An ideal anti-malarial drug should target all stages of the parasite life cycle. One such vulnerable aspects of *Plasmodium* lifecycle could be the DNA double strand break (DSB) repair pathway. Since, DNA damage occurs in every stages of parasite life cycle the failure to repair DNA double strand breaks would lead to lethality. Moreover, abrogation of DSB repair resulted in less virulent parasites (Roy et al. 2014).

1.2. DNA double strand break and its repair in eukaryotes:

DNA damage is inevitable as it arises during normal cellular processes. Unicellular and multi-cellular organisms are continuously exposed to various agents that cause DNA damage (Smeenk et al. 2013). Majorly of DNA damage is caused either by exogenous sources like ionizing radiation or by endogenous sources like free radicals that are generated during metabolism. DSB might also arise due to the collapse of replication fork or due to dysfunctional nuclear enzymes and programmed DNA breaks (Figure 2) (Lieber et al. 2010). Eukaryotes have evolved with protective mechanisms to maintain genome integrity. In unicellular organisms, if the DNA breaks are not repaired that leads to lethality.

DNA breaks can be categorized in two types, single standard breaks (SSBs) and double standard breaks (DSBs) (Figure 3). Unrepaired SSBs lead to DSBs during S-phase of the cell cycle, whereas, in other phases of cell cycle and in non-proliferating cells, accumulation of SSBs leads to stalling of transcription (reviewed in Caldecott et al. 2008). SSBs can be repaired by three major DNA repair pathways; base excision repair (BER), nucleotide excision repair (GG-NER & TC-NER) and mismatch repair (MMR). Defects in SSB repair genes cause genetic diseases in humans, for example Xeroderma Pigmentosum (XP) and Cockayne's Syndrome (CS) (reviewed in Driscoll et al. 2012).

DNA double-strand breaks (DSBs) are the most severe form among all kinds of DNA damage. In general, DSBs are produced during generation of free radicals and during arrested replication fork processing. Programmed DSBs are generated by nucleases in different systems, such as in mating type switching in yeast, during V(D)J recombination (RAG1, 2) and during class switch recombination (AID/UNG/APE). Chromosomal rearrangements and fusions that occur due to the failure of telomere maintenance might leads to DSBs in cancerous cells (reviewed in Shrivastav et al. 2008, Lieber et al. 2010). Unrepaired or inaccurate repair of such breaks lead to genome instability and cause genetic disorders such as Ataxia Telangiectasia, Nijmegen breakage

syndrome, Bloom's syndrome and Werner's syndrome. Even a single unrepaired DSB would be lethal for the survival of unicellular organisms (Frankenberg-Schwager et al. 1990). In eukaryotes, DSB mediated DNA damages are repaired by two major repair pathways: a) Non-homologous end joining repair pathway (NHEJ); b) Homologous recombination (HR). NHEJ involves direct ligation of two broken ends, which does not require homologous sequence (Figure 4). Recently an alternative pathway called micro-homology mediated end joining (MMEJ) has been discovered which requires micro homologous sequence at the broken ends. HR requires homologous sequence as a template to repair broken ends, which is an error-free repair pathway.

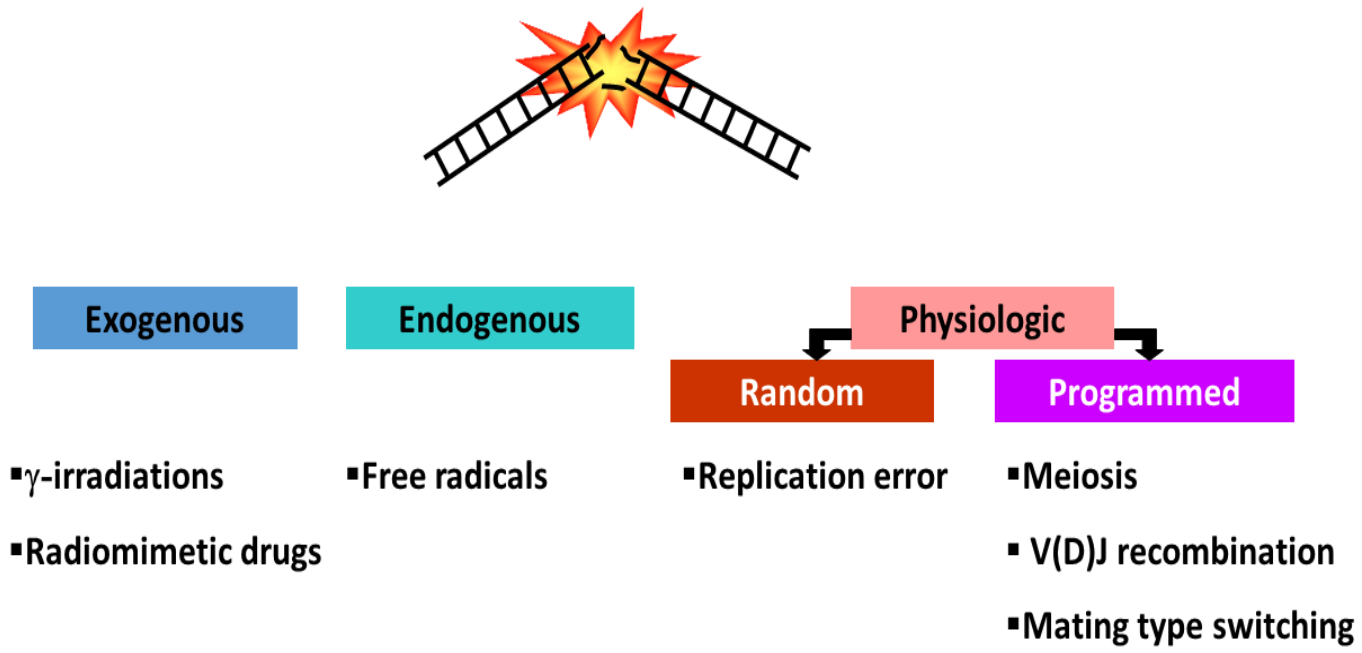


Figure 2: Illustration of possible sources for inevitable DNA damage.

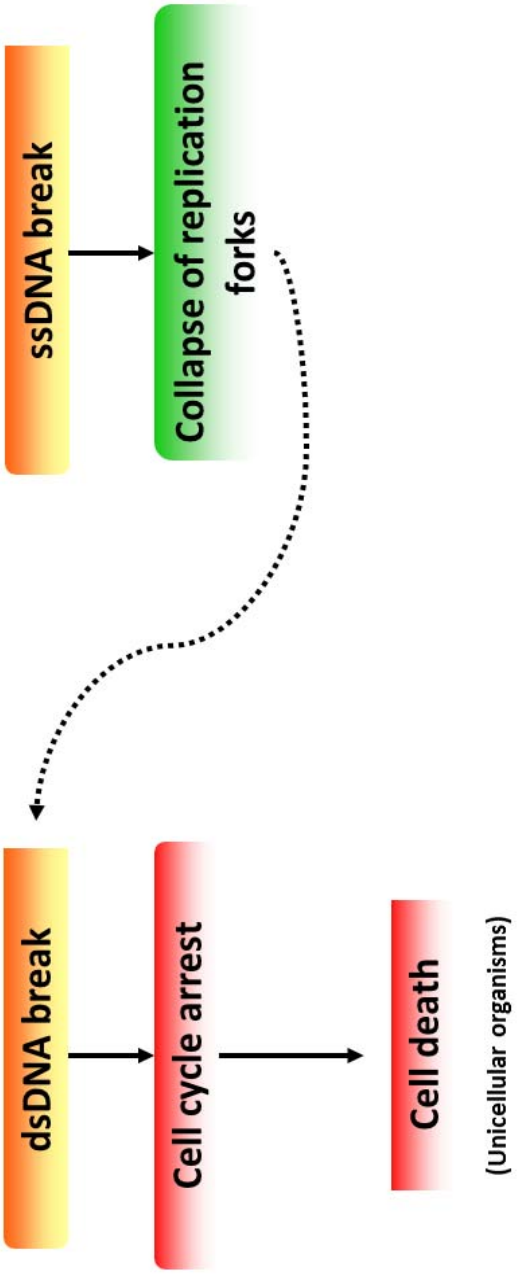


Figure 3: Consequences of single strand breaks and double strand breaks in unicellular organisms.

1.2.1 DNA damage signaling cascade and cell fate:

Cells are evolved with sophisticated signaling network which senses DNA damage and arrests cell cycle to activate DSBs repair pathways. DNA damages generated during cellular processes or by external agents are detected by sensor proteins which subsequently activate the signaling cascade. Depending upon the type of the damage, the transducers/mediators activate downstream effector molecules (Figure 4). It is shown that initial steps in detecting and responding to the DNA damage occur by recruitment of the MRX complex (Mre11-Rad50-XRS2/NBS1) which amplifies the signal through initial processing of DSB ends (reviewed in Lavin et al. 2007). Accumulation of ssDNA signals is amplified by RPA/Rfa and PCNA like clamp protein complex. Phosphoinositide 3-kinase related (PIKK) family kinases such as ATM and ATR also play crucial role in signaling cascade. MRX/N complex activates ATM (ataxia telangiectasia mutated) kinase and RPA contributes to the recruitment of ATR (ATM and Rad3-related) along with ATRIP (ATR interacting partner). These transducers activate specific effector pathways in association with mediator proteins (Polo et al. 2011) (Table 1). The effector kinases are recruited at the DSB sites and depending upon the state of the damage, they activates the cascades of proteins like transcription factors, cell cycle checkpoints regulators, proteins involved in apoptosis machinery and that involved in DNA repair pathways (Polo et al. 2011).

DNA damage arrests the cell cycle progression at either of the cell cycle checkpoints: G1/S checkpoint before DNA replication, S-phase during replication and G2/M checkpoint before mitotic phase. Cells traversing through G1 phase with damaged DNA activate G1/S checkpoint through ATM/ATR and CHK1/CHK2, which in turn arrest the cell cycle by activation of transcription factors p53 and cdc25A. ATM gets activated by the nuclease activity of MRN/X complex which subsequently causes cell cycle arrest through cdc25A mediated de-phosphorylation and NBS1/SMC1 phosphorylation. ATM/ATR and CHK1 also cause G2/M checkpoint arrest by inactivation of cyclin B/cdk1 (reviewed in Lukas et al. 2004). Cell cycle

arrest allows the cell to repair the damaged DNA by one of the repair pathways (Lukas et al. 2004). All these DNA damage repair activities are collectively known as DNA damage response.

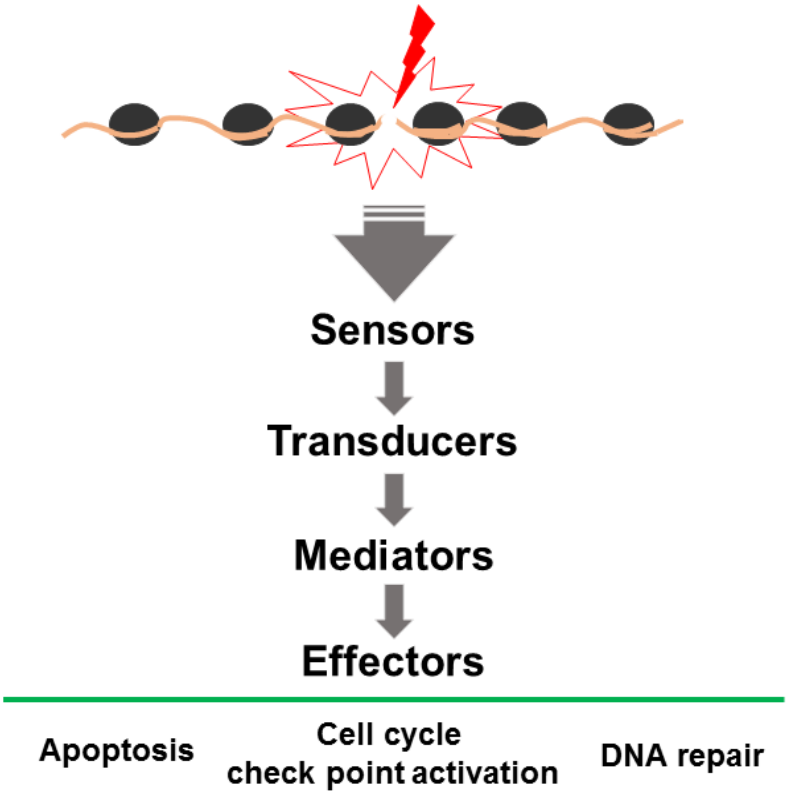


Figure 4: Illustration of DNA damage response pathway.

Table 1: Proteins involved in DDR in eukaryotic model organisms

DNA damage response cascade components	Mammals	Yeast (*S. cerevisiae*)
Sensor	MRN RPA (+RFC like, PCNA-like check point clamp)	MRX Rfc ((+RFC like, PCNA-like check point clamp)
Transducers	ATM ATR-ATRIP	Tel1 Mec1-Ddc2
Mediators ATM signaling ATR signaling	 53BP1, MDC1, BRAC1, MCPH1, PTIP TOPBP1 Claspin	 Rad9 Dpb11 Mrc1
Effectors	CHK1 CHK2	Chk1 Rad53

1.3 DNA DSBs repair pathways:

Two major DNA repair pathways attribute to repair of DSBs. They are:

a) Non-homologous end joining repair (NHEJ) which is an error-prone repair pathway.

b) Homologous recombination (HR) which is an error-free repair pathway.

1.3.1 Non-homologous end joining repair pathway (NHEJ):

NHEJ is defined as rejoining of two broken ends independent of any homologous template. This type of repair mechanism is found to be present in prokaryotes as well in eukaryotes. All the components of the pathway arose independently, except for the Ku proteins. The pathway is well established in *S. cerevisiae* and in humans (where it is the major DSBs repair pathway). NHEJ is found to be active throughout the cell cycle and is more prominent in G1 phase upon DNA damage (Smeenk et al. 2013). In vertebrates, NHEJ is initiated through binding of Ku proteins to the broken ends of DNA. Ku protein-DNA complex recruits nuclease, polymerase and ligase in a flexible manner independent of each other. This flexibility of recruitment allows differential outcome products during V(D)J recombination. Briefly, the classical NHEJ is initiated by binding of Ku70-Ku80 heterodimer protein complex on broken DNA end, which subsequently recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and activates its protein kinase activity. The activated DNA-PKcs recruits and activates the endonuclease activity of Artemis to trim the broken ends. DNA pol μ/ λ complex fills the gaps and finally the ends are ligated with the help of XLF-XRCC4-ligase IV complex (Iyama et al. 2013). This pathway is well known as classical NHEJ or cNHEJ.

Cells which are deficient in cNHEJ can repair DSBs by another pathway known as alternative (Alt-NHEJ)/ micro-homology end joining pathway (MMEJ). In this pathway, two broken ends are ligated by a micro-homology sequence generated by MRN/X complex and it is a Ku protein independent process. This pathway was identified in yeast and mammals as a backup pathway for cNHEJ. Recent studies showed that Alt-NHEJ could repair 10% of DSBs in cNHEJ proficient

organism even in the absence of cNHEJ factors. Alt-NHEJ is carried out by end processing nuclease activity of Mre11 and CtIP. The micro-homology ends that are generated by this process are proficient for ligation by DNA ligase III/DNA ligase I. Further, recent work suggests that PARPI and DNA ligase III play critical roles in Alt-NHEJ at telomeres (Figure 5). (reviewed in Deriano et al. 2013).

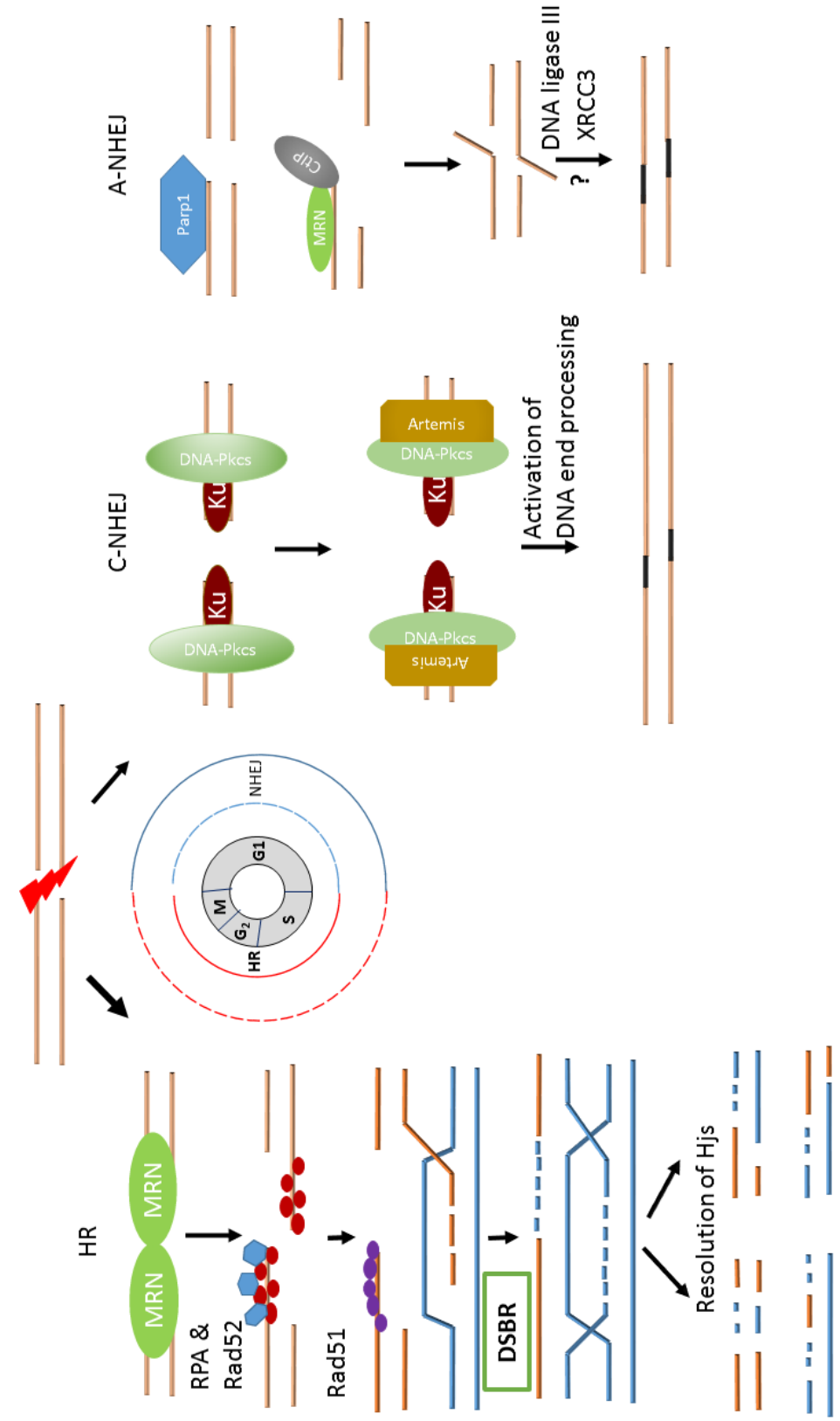


Figure 5: DNA double strand break repair mechanisms (Iyama T et al., 2013 & Deriano et al., 2013). Some of the key proteins are shown.

1.3.2 Homologous Recombination (HR):

HR is an error free DSBs repair pathway where homologous DNA sequences is used as a template to repair the broken ends. It is evolutionarily conserved in prokaryotes and eukaryotes. During meiosis HR generates diversity in the progeny through exchange of genetic information between the paternal and maternal alleles. HR mediated repair pathway occurs in S and G_2 phases of the cell cycle, where sister chromatids are generated (reviewed in Smeenk et al. 2013). HR genes involved in the repair of DSBs were identified by mutations that showed hypersensitivity towards DNA damaging agents. Based on the HR repertoire participation in the repair of DSBs, HR pathways are classified into four models: a) double strand break repair (DSBR); b) synthesis dependent strand annealing (SDSA); c) single strand annealing (SSA); and d) break induced replication (BIR) (Figure 6). In DSBR model, the repair process is initiated by the nucleolytic resection at the broken junction to produce 3' over hangs, which acts as a template to form recombinase filament on ssDNA. This process is further followed by the formation of strand invasion into a homologous sequence to form D-loop. DNA polymerase extends the sequence from 3' end which allows the formation of Holliday junctions (HJ). Finally, the HJs are resolved to give crossover products. This model can explain the consequences of meiotic recombination. SDSA is also operational in meiosis and mitotic cells and the end product always generate non-crossover products. If the DSBs are flanked by direct repeat sequences, DSBs are repaired by the deletion process called single strand annealing (SSA). This process initiates at the broken junction and is independent of the strand invasion and HJ formation. The mechanism allows the deletion of sequence between the direct repeat sequences. Break induced replication can be operational at one of the broken ends, which occurs at the telomere ends and near stalled replication forks (Figure 6) (Reviewed by Heyer et al. 2008 & Symington et al. 2002).

The genes in HR are characterized in *S. cerevisiae* by epistasis analysis. The *RAD52* epistasis genes were divided into three groups: Class I consisting of: *RAD50*, *MRE11* and *XRS2*; class II

including: *RAD51*, *RAD52* and *RAD54*; and class III consisting of *RAD55*, *RAD57*. HR pathway broadly operates in three steps: pre-synopsis, synopsis and post-synopsis. In pre-synopsis, DSB ends are processed by MRX/N complex, *EXOI*, *DNA2*, *SAE2* (CtIP in humans) and *SGS1* helicase (BLM) to generate 3' overhang ssDNA. This allows RPA protein to bind the overhangs and the formation of Rad51 filament is mediated by Rad55, Rad57 and Rad52 (BRCA2 in humans). In synopsis, Rad51 protein searches for the homologous sequence and facilitates strand invasion which leads to the formation of D-loop. Rad54 protein acts as a motor to stabilize Rad51 filament for D- loop extension and allows the DNA synthesis in hetero-duplex. In post synopsis phase, hetero-duplex is processed by any of the three models (SDSA, BIR & HJs). Finally, the HJs are resolved by endo-nucleases and RecQ helicases family of proteins (reviewed in Heyer et al. 2010, San Filippo et al. 2008).

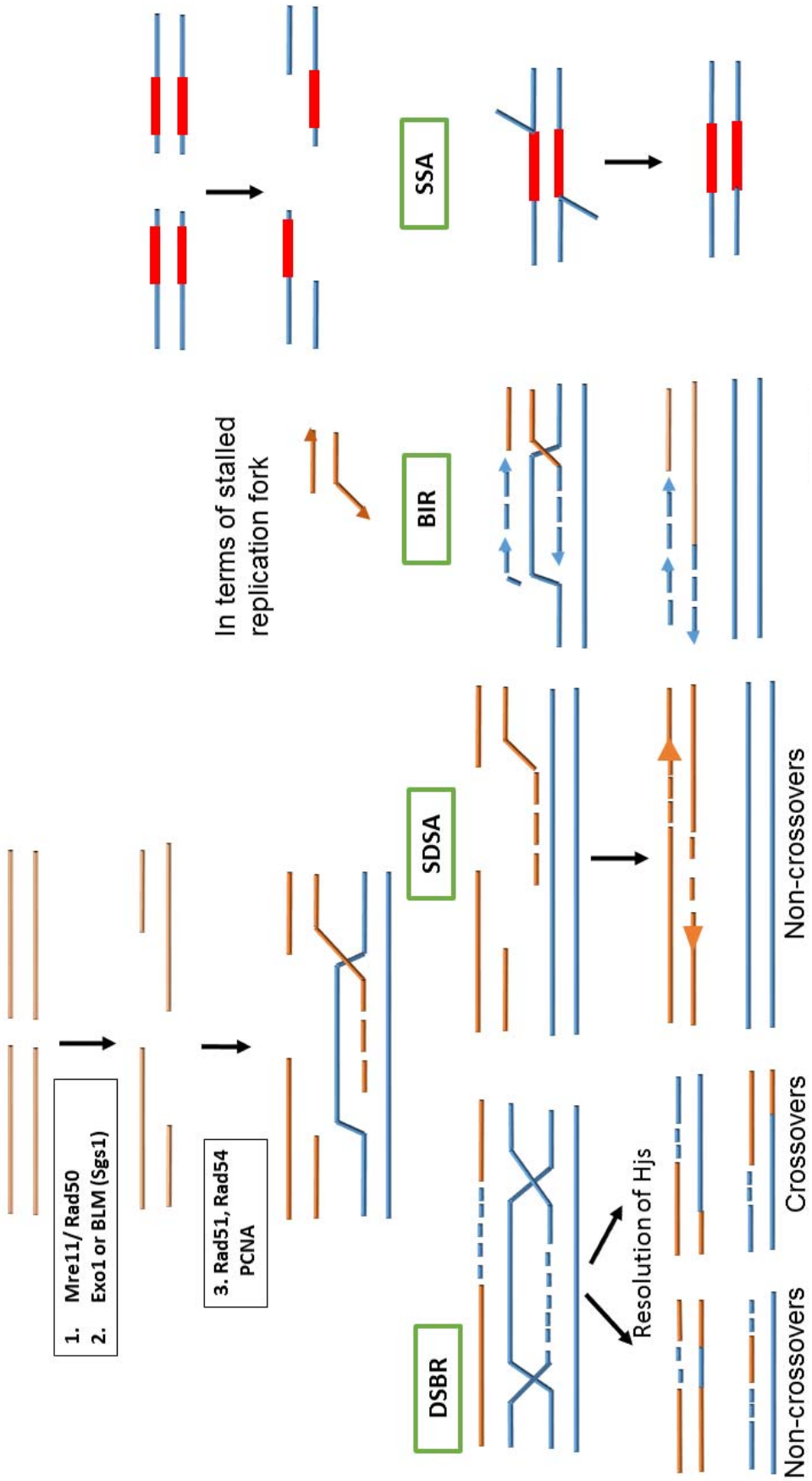


Figure 6: Homologous recombination models.

1.4 DNA repair mechanisms in apicomplexan parasite *Plasmodium falciparum*:

Plasmodium genome sequence analysis revealed the presence of homologues genes in NER and BER pathways of DNA repair (XPB/Rad25=PF3D7_1037600, XPG/Rad2=PF3D7_0206000, XPD/Rad3=PF3D7_0934100, ERCC1=PF3D7_0203300, AP-endonuclease) (Gardner et al. 2002, Bhattacharyya et al. 2004). BER in *P. falciparum* has been reported to repair the DNA lesions by long patch BER pathway (Haltwanger et al. 2000). One of the BER components, Uracil- DNA glycosylase was characterized as an antimalarial drug target (Suksangpleng et al. 2014). *In silico* analysis of NER components suggested the presence of all the homologs except for p62 and XPC (Tajedin et al. 2015). In case of mismatch repair (MMR) pathway putative orthologues of UvrD helicase was identified in *Plasmodium* and its functions was explored by complementation and knockout studies (Bethke et al. 2007) and by biochemical approaches (Tarique et al. 2012, Ahmad et al. 2014). Drug resistance parasite strains such as W2 and Dd2 weres found to possess inefficient MMR mechanism. Studies using forward genetics in W3, Dd2 and HB3 strains revealed that the inefficiency in MMR could be the cause of multidrug resistance (Castellini et al. 2011).

1.5 Double-strand break repair mechanism in *P. falciparum*:

Apicomplexan parasites undergo mitotic divisions at three stages (sporozoites production in oocyst, liver stage, and erythrocyte schizont stage) and meiosis in zygote. The parasites are exposed to various DSB causing agents through all phases of development. The merozoites invaded RBC develops into ring stage (0 hr – 24 hrs), which corresponds to the G1 phase of cell cycle. Any DSB's which occur at this stage must be repaired by non-HR mechanism as the parasites possess haploid genome at this stage. Parasite transitions to trophozoite and schizont

stages are considered as S/G2/M phase. Here parasite DNA is replicated and thus DSBs could be repaired via HR mechanism utilizing the unbroken sister chromatids (Figure 7).

1.5.1 Non-homologous end joining:

Plasmodium genome sequence analysis could not reveal any ortholog for classical NHEJ proteins (Gardner et al. 2002). However related apicomplexan parasite *Toxoplasma gondii* possesses most of the components of classical NHEJ pathway. Other eukaryotic pathogens such as *Giardia lamblia, Encephalitozoon cuniculi, Trichomonas vaginalis* seem to lack the classical cNHEJ components (Lee et al. 2014). Reports from another parasite *Trypanosomes* are known to have both the HR and cNHEJ mechanisms (reviewed in Passos-Silva et al. 2010). It has been suggested that during evolution, *Plasmodium* might have lost the NHEJ pathway. A recent study has suggested the existence of the Alt-NHEJ mechanism in *P. falciparum* which is used at a very low frequency compared to the HR mechanism (Kirkman et al. 2013).

1.5.2 Homologous recombination mechanism in *Plasmodium falciparum*:

Homologous recombination occurs in *Plasmodium* during the sexual reproduction of the parasite inside mosquito mid-gut. It is also observed during MSP-1 gene rearrangement, multi-drug resistant gene *PfMDR* rearrangements and during spontaneous gene rearrangements at the *Var* gene cluster of the parasite. Apart from this as *Plasmodium* is constantly exposed to free radicals during heme metabolism, it generates various DSBs which are expected to be repaired by HR. Several of the proteins involved in HR (Mre11, DMC1, Rad50, Rad54, RPA, and Rad51) were identified in the genome sequence analysis (Gardner et al. 2002). PfRad51 level was found to be increased in *P. falciparum* culture upon MMS treatment. The ATP hydrolysis and strand exchange activities of PfRad51 were characterized through biochemical studies (Bhattacharyya et al. 2003 & 2005). Mutant parasites with loss of HR function were found to be hypersensitive to DNA damage and were also less virulent (Roy et al. 2014). Other HR proteins like PfRad54 and PfRPA1 variant were also biochemically characterized (Gopalakrishnan et al. 2013, Voss et al.

2002). *Plasmodium* possibly does not possess Rad52 as Rad52 ortholog could not be identified in the parasite genome sequence. However, BRCA2 has been identified in *Plasmodium* (Lee et al. 2014). RecQ orthologs WRN and BLM are functionally characterized in *Plasmodium* (Rahman et al. 2015). PfRecQ1 which is human ortholog of RecQ possesses DNA unwinding activity (Suntornthiticharoen et al. 2014).

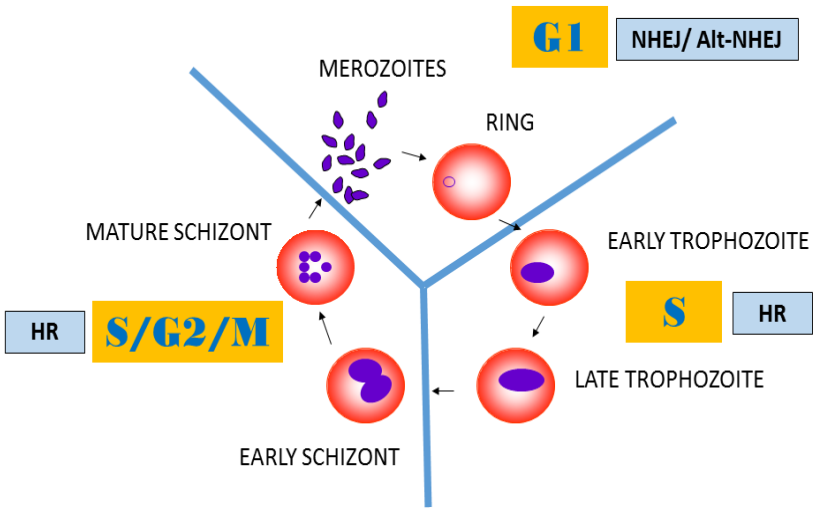


Figure 7: Probable DSBs repair mechanisms during intra-erythrocytic growth of *P. falciparum*.

1.6 Mre11: Key molecular player in DNA damage response pathway:

Functional role of Mre11p in the repair of DNA double-strand breaks has been well studied in *S. cerevisiae*. Mre11p complex plays a crucial role in the maintenance of genome integrity, during the release of arrested DNA replication forks, DNA double strand break repair, telomere length maintenance and DNA damage signaling (Amours D' et al. 2002; Assenmacher et al. 2004). Mre11p participates in DNA double strand break repair by forming a functional complex Mre11/Rad50/Xrs2 (NBS1 is the ortholog of Xrs2 in human) in the ratio of 2:2:1 (reviewed in Haber et al. 1998, Usui et al. 1998). Mre11 and Rad50 proteins are evolutionarily conserved whereas Xrs2/NBS1 shows variation in sequence. However together they possess a conserved DDR function (reviewed in Amours D' et al. 2002). Deletion of any one of the genes in the complex is lethal in human. In yeast deletion of any component of this complex leads to poor cell growth whereas in mammals it leads to embryonic lethality (Xiao et al. 1997). The Mre11 complex plays two distinct roles: a) Mre11p processes the broken DNA ends using its exonuclease and endonuclease activities b) MRN/X complex participates in the activation of DNA damage signaling pathway. The Mre11p complex participates in DNA double-strand break (DSB) repair through either of the two DNA repair pathways- HR and NHEJ. MRX complex also plays a role in meiotic recombination (Haber et al. 1998, Paques et al. 1999). The two distinct functions are associated with N terminal and C terminal domains of Mre11p (Figure 8) (Usui et al. 1998; Furuse et al. 1998). Mre11p participates in DNA damage signaling as an upstream DNA damage sensor protein and acts as a mediator/effector for activation of ATM (Tel1p in yeast) (Amours D' et al. 2002). It activates ATM (Tel1) through phosphorylation and ATM also phosphorylates Mre11p and Xrs2/NBS1 in response to DNA damage. ATM mediated phosphorylation of Mre11p releases the activation of DNA damage signaling and the damaged DNA gets repaired (Virgilio et al. 2009). In mitotic cells, DSBs signaling is amplified by two parallel events, the TM (Tel1-Mre11p) pathway and the Mec1 pathway. In meiotic cells, TM

pathway is dependent on un-resected DSBs but not on unrepaired DSBs (Usui et al. 2001). Studies on humans and yeast claim that MRX/N complex is recruited to the telomeres (Takata et al. 2005, Zhu et al. 2000). Deletion of the above mentioned genes causes telomere length shortening (Boulton et al. 1998, Nugent et al. 1998) and telomere fusion (Bi et al. 2004). Tel1 interacts with Mre11 and participates in DNA damage response activity. The C-terminal domain of Mre11 participates in DNA damage signaling by interacting with Xrs2. The nuclease activity of Mre11 provides protection to the telomeres (Bhattacharyya et al. 2008). Double mutant of *mre11 tel1* leads to end-to-end fusions and gross chromosomal rearrangements (Ritchie et al. 2000; Myung et al. 2001). In humans it has been observed that the DNA damage foci at the dysfunctional telomeres contains hMre11, ATM (Tel1) and other DNA damage response factors (Takai et al. 2003) which suggests that the telomere protection is mediated by Tel1p (ATM).

Mre11p activates DNA repair with its N-terminus nuclease activities (Amours D' et al. 2002). MRX complex is essential for joining the incompatible ends during NHEJ and the ATP-dependent activity of Rad50 is critical for this process (Zhang et al. 2005). Mre11 complex is required for telomere length maintenance in two ways; firstly it produces single stranded telomeres which are prerequisite for telomerase loading during DNA replication (Diede et al. 2001). Secondly it is involved in recombination mediated telomere length maintenance (Chen et al. 2001). MRX complex activates the DNA damage signaling pathway *via* Tel1 in yeast by forming Tel1- Mre11 complex which participates in cell cycle checkpoint signaling pathway designated as TM pathway.

Mre11p participates in DNA repair activity with its intrinsic nuclease activity which is activated in presence of Rad50 (Stracker et al. 2004). The nuclease domain of Mre11 is associated with four phosphodiesterase motifs, located at its N-terminus. Similar domain was found in *E. coli* SbcD subunit of SbcCD complex (Connelly et al. 2002). In yeast and human it has been demonstrated that Mre11p acts on double-stranded DNA in 3' to 5' direction and cleaves single strand DNA with its intrinsic endonuclease activity which is Mn^{+2} dependent (reviewed in Haber

et al. 1998). *In vitro* studies have demonstrated that Mre11P exonuclease activity is structure specific and it is active on blunt end DNA, on hairpin structures and on 3' resected DNA products (Amours D' et al. 2002). In yeast, genetic and biochemical studies revealed that Mre11p can bind to the G quadrate structures and generates appropriate substrates for DNA synthesis (Ghosal et al. 2005, Larrivee et al. 2004). Mre11p endonuclease activity at telomere ends is regulated by ATPase activity of Rad50. Rad50 negatively regulates the endonuclease function of Mre11 towards non-specific cleavage of telomeric and genomic DNA (Ghosal et al. 2007).

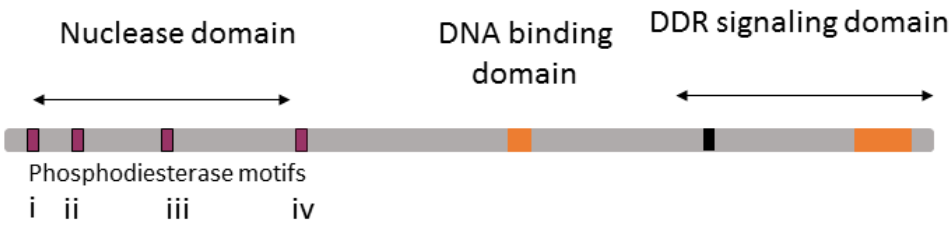


Figure 8: Illustration of functional domains of Mre11 protein.

The nuclease functions attributed to the phosphodiesterase motifs are confirmed by mutational analysis. The *mre11D16A* abrogates the nuclease function in mitotic cells whereas the deletion mutant *mre11Δ49C* (at C-terminal domain) does not show any effect on the nuclease activity although it interferes with the DSB formation during meiosis (Furuse et al. 1998). Other nuclease mutations of mre11 namely D56N, H125N, H213Y (mre11-58) are found to reduce nuclease activity demonstrating that Mre11 D16, D56, H125 and H213 residues are critical for nuclease functions. The nuclease functions at un-resected DSBs are processed by Mre11p complex along with other nucleases. Sae2, Exo1, Sgs1 and Dna2 are found to be active on DSBs in mitotic and meiotic cells. Sae2 endonuclease activity is demonstrated on hairpin DNA structures. It is observed that the deletion of Sae2 along with MRX complex fails to process hairpin like structure that occurs during brake induced repair mechanism (Lengsfeld et al. 2007). Mre11p complex processes the double strand breaks to generate short 3' over hangs which are extended by the 5'-3' exonuclease activity of EXO1. Studies on *Δexo1* mutant exhibited variation in length during DNA end resection (Llorente et al. 2004, Moreau et al. 2001) suggesting that Exo1 is a redundant protein of Mre11p complex. In a parallel pathway, RecQ helicase Sgs1 is found to be involved in the resection of double strand breaks along with Dna2 (reviewed in Mimitou et al. 2009). Mre11p is an upstream DNA damage repair protein in both the double strand break repair pathways namely HR and NHEJ. It is still a puzzle that how cell chooses a repair pathway. It is believed that repair molecules might be responsible for triggering the pathway that is dependent on the cell cycle. Recently it has been demonstrated that Mre11 nuclease activities might be responsible for the pathway choice. It has been shown that the inhibition of exonuclease activity of Mre11 and activation of its endonuclease function initiates NHEJ (Shibata et al. 2014).

DNA damage repair is evolutionarily conserved in eukaryotes even though, very less is known about the repair pathways in protozoans, early eukaryotes. *Trypanosoma brucei* Mre11 homolog is

identified and functionally characterized its role DNA damage repair. A conditional mre11$^{-/-\ (+)}$ mutant shows reduced growth rate, a similar phenotype is observed with yeast Me11 and hypersensitive to DNA damage inducing agents. The Mre11 null mutant of *T. brucei* essentially affects homologous recombination thereby genome stability but not the VGS recombination, a mechanism similar to antigenic variation which escapes the host immune system (Tan et al. 2002 & Robinson et al. 2002). Ku and Mre11 protein complex play essential role in the maintenance of telomeres in yeast and humans however, *T. brucei* telomeres are independent of Mre11 but require Ku protein (reviewed in Dreesen et al. 2007).

1.7 SIGNIFICANCE OF THE STUDY:

DSBs are the most lethal form of DNA damage. Unrepaired or inaccurate repair of such damage leads to genome instability and cause genetic disorders like ataxia telangiectasia, Nijmegen breakage syndrome, Bloom's syndrome and Werner's syndrome. A single unrepaired DSB would cause the death of unicellular organisms (Frankenberg-Schwager et al.1990). Since *P. falciparum* is a unicellular organism, targeting DDR molecules would be a good strategy to eradicate malaria. DDR orthologs are identified in *Plasmodium* genome, but they need to be characterized in order to use them as drug targets. In this perspective, initially we have studied the efficiency of DSB repair mechanisms in different *P. falciparum* strains, geographically distributed in different regions. We found that DSB repair mechanisms are effective among drug resistant and sensitive parasite strains distributed in different geographical regions. Mre11 is an early sensor protein recruited at DSB ends. PfalMre11 a putative ortholog of Mre11 protein is identified in genome annotation. The presence of less homology sequence similarity with humans Mre11 ortholog allows to check for drug target, as it was found to be essential in other organisms. PfalMre11 is up-regulated upon DNA damage induction in *in vitro* parasite culture, suggest that PfalMre11 might be having a functional role in DDR pathway. We have characterized its functional roles using yeast surrogate system. Yeast two-hybrid analysis with genomic library given the clues to the presence of DNA damage response pathway molecules and the studies can be extended to find out DNA damage response molecules in *P. falciparum*.

These observations elicit to study the DDR proteins to use as drug candidates. Since PfalMre11 is essential DSB repair protein, targeting this protein could block both the DSB repair pathways; homologous recombination pathway (HR) and alt-NHEJ.

1.8 OBJECTIVES OF THE STUDY:

P. falciparum is a unicellular apicomplexan parasite. Unrepaired DNA damage leads to accumulation of lesions thereby challenging the survivability of the parasite. DSB are the most lethal forms of the DNA damage. Previous studies in *P. falciparum* shows that homologous recombination (HR) is the predominant pathway (Roy et al.2014), whereas Alt-NHEJ is less preferred (Krikman et al. 2013). Previously it was reported that parasite strains, resistant to chloroquine, are defective in DNA damage repair (Trotta et al. 2004). To study the DSB repair mechanisms in detail we aimed to characterize key molecular players of DDR in *P. falciparum*. In this work, three questions have been addressed. We asked whether drug resistant and sensitive *Plasmodium* parasite strains are differentially sensitive to DNA damage agents and can repair the DNA damage. Secondly we have asked whether *Plasmodium* possesses a functional ortholog of Mre11, a predominant DDR protein. Thirdly what are the molecular interactors of PfalMre11.

1.9 AIMS OF THE PhD WORK:

1. Study of DNA damage repair efficiency in drug sensitive and resistant parasite strains: we have used *P. falciparum* strains 3D7 (drug-sensitive) and Dd2 (drug-resistant) to analyze the DNA damage response and the repair efficiency at nuclear and mitochondrial genomes.

2. Characterization of putative Mre11 ortholog in *P. falciparum*: We have used *P. falciparum* in vitro culture and yeast as a surrogate system to study the functional activities of PfalMre11 upon DNA damage induction.

3. Genome-wide screening of *P. falciparum* genome library to identify PfalMre11 interactor: We have used yeast two-hybrid system to screen the PfalMre11 interactor.

MATERIALS AND METHODS

2.1 Recombinant DNA methods:

2.1.1 Bacterial competent cell preparation:

Primary inoculum was given in 5 ml of LB medium for overnight at 37°C incubator. 1 ml of the primary culture was then used as inoculum for secondary culture which was incubated at 37^0C until the OD_{550} reached 0.5 to 0.7. Bacterial culture was harvested by centrifugation at 8000 rpm, 4^0C for 8 minutes. The cell pellet was gently mixed with half volume (25 ml) of ice cold 0.01M $CaCl_2$. Again the culture was pelleted down by centrifugation at 8000 rpm, 4°C for 8 minutes. The pellet was then resuspended in 0.25 volumes (12.5 ml) of ice cold 0.01M $CaCl_2$ and incubated on ice for 4 hrs. The culture was centrifuged at 8000 rpm, 4°C for 8 minutes and resuspended in 2.15 ml of ice cold $CaCl_2$. The pellet obtained was mixed with 350 µl of Glycerol. 100 µl of the competent cells were aliquoted into pre-chilled microcentrifuge tubes and frozen in liquid nitrogen followed by storage at -80°C.

2.1.2 Bacterial transformation:

The competent cells were thawed to which approximately 50 ng of plasmid DNA was added and incubated for 30 minutes on ice. The cells were then given heat shock at 42°C for 30 seconds and immediately transferred to ice for 2 minutes. 900 µl of LB medium was added to the cells and incubated at 37°C incubator with 200 rpm for 1 hr. The samples were then pellet down by short spin and most of the supernatant was discarded. The cell suspension was spread on LB agar plates containing appropriate antibiotic followed by incubation at 37°C incubator for 16 hrs.

2.1.3 Plasmid DNA isolation by alkaline lysis method:

Bacterial cells harboring plasmid DNA were inoculated in 5 ml of LB medium with appropriate antibiotic. The culture was incubated for overnight at 37°C incubator. The cells were harvested by centrifugation at 4000 rpm for 5 minutes and resuspended in pre-chilled 200 µl of solution 1 (25 mM Tris pH 8.0, 10 mM EDTA pH 8.0). The suspension was transferred to a microfuge tube and 200 µl of solution 2 (0.2 M NaoH, 1% SDS) was added after which the tube was inverted for several times and incubated at room temperature for 5 minutes (do not vortex). 150 µl of solution 3 (3 M NaOAc pH 5.2) was added to the viscous solution and mixed well by inverting for several times and incubated on ice for more than 5 minutes (formation of white precipitate could be observed during this step). The sample was centrifuged at 12000 rpm for 15 minutes at room temperature and the supernatant was collected into a new microfuge tube. 2.2 volume of 100% alcohol was added to the supernatant and incubated at -20°C for 45 minutes. The samples were then centrifuged at 12000 rpm, 4°C for 30 minutes to precipitate the plasmid DNA. The pellet obtained was washed with 70% alcohol by centrifugation for 5 minutes at 12000 rpm, 4°C. The pellet was air dried and resuspended in 30 µl of 1X TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) to which 5 µl of RNase (10 mg/ml) was added and incubated for 30 minutes. The sample volume was made up to 400 µl by adding 1X TE, equal volume of PCIA (Phenol: Chloroform: Iso amyl alcohol) was added and vortexed for 3 minutes until the sample became homogenous. The sample was centrifuged at 12000 rpm for 15 minutes. The aqueous layer was aliquoted in a new tube and DNA was precipitated by adding $1/10^{th}$ volume of solution 3 and 2.2 volume of 100% alcohol and incubated at -80°C for 2 hrs (or at -20°C for overnight). The plasmid DNA was pellet down by centrifugation at 12000 rpm, 4°C for 30 minutes. The supernatant was discarded and the pellet was washed with 500 µl of 70% alcohol. The pellet was then air dried and resuspended in 30 µl of 1X TE buffer. Plasmid DNA quality was checked by agarose gel electrophoresis.

2.1.4 Site- directed mutagenesis by overlap PCR method:

The list of primers used to generate different mutant versions of *PfalMRE11* are listed in Table 2. ORF was PCR amplified into two fragments using one of the primers without mutation and other primer having base mutation. Then the full length ORF was PCR amplified using both the fragments having induced mutation and full length ORF primers, where internal overlap sequence having mutation served as primer to extend the template to get the full-length sequence. The final PCR product was cloned into InsTA vector pTZ57R/T (Thermo Scientific) and the ligation mixture was transformed into bacterial competent cells and the clone was confirmed by restriction digestion. The mutation region was sequenced by sequencing primer to confirm the introduced mutation.

2.1.5 Method for *Plasmodium* genomic DNA library construction:

Plasmodium genome was isolated by standard protocol and put for restriction digestion by BamHI enzyme. 10 μg of library preparation vector pGADC1 (U70024) was isolated by alkaline lysis method and linearized by BamHI restriction digestion followed by phosphatase treatment. The purity of the linearized vector was confirmed by checking self-ligation of the plasmid through bacterial transformation. Expected colony number was calculated for the average size of the fragments generated by BamHI by following the formula

N= ln (1-P)/ ln (1-a/b). Where N= no of colonies required, P = probability that given gene will present, a= average size of DNA fragments inserted into vector, b= total size of genome (*P. falciparum* genome size 23 Mb) and total plasmid DNA concentration was calculated to set up the ligation reaction. The ligation sample was kept at 16°C for 12 hrs. The total ligation mixture was transformed into high competent cells and the total library was isolated by maxi prep protocol (QIAGEN). The library was stored in -20°C for the further purpose.

2.2 Yeast methods:

2.2.1 Yeast competent cell preparation:

A freshly revived yeast strain was inoculated in 5 ml of the medium as primary inoculum and incubated in 30°C incubator shaker at 200 rpm. Next day the primary inoculum OD_{600} was measured after 1:10 dilution. The total volume of the culture for secondary inoculum was calculated by following formulae:

Volume of inoculum = (Final volume of secondary culture x 0.5 OD)/ (Initial OD_{600} x 2n, n= no of generations, usually 2 generations). Secondary inoculum was incubated at 30°C, 200 rpm to reach the required OD_{600} 0.6-0.8. The culture was pelleted by centrifugation at 4°C, 3500 rpm for 5 minutes. The culture was washed by resuspending in 10 ml of Milli-Q water (autoclaved) and centrifuged at 4°C, 3500 rpm for 5 minutes. The cell pellet was finally resuspended in 300 µl of lithium acetate solution (1X TE, 1X LiOAc & Milli-Q) and hence the competent cells were used for transformation.

2.2.2 Yeast transformation:

1-3 µg of target plasmid DNA aliquot was mixed with 10 µg of single-stranded carrier DNA in a micro-centrifuge tube. 200 µl of yeast strain competent cells were added to the DNA mix in the laminar hood. Cell were then mixed with 1.2 ml of PEG solution (for 1 ml, 100 µl of 10X TE, and 100 µl of 10X LiOAc and 800 µl of 50% PEG2000 solution) and incubated for 30 minutes in 30°C incubator shaker at 200 rpm. Later the cells were exposed to heat shock for 15 minutes at 42°C. Immediately samples were transferred to ice for 2 minutes. The cells were then pellet down by short centrifugation and resuspended in 200 µl of 1X TE buffer. The suspension was spread over the agar plates and incubated at 30°C incubator for 72 hrs.

2.2.3 RNA isolation from yeast strain:

Yeast cells were grown in 10 ml media of choice to mid-log phase (OD_{600} 1.0). Cells were pelleted by centrifugation for 5 minutes at 3500 rpm. The pellet was resuspended in 500 µl of DEPC ddH_2o and harvested by short spin at 10000 rpm. The supernatant was discarded and the pellet was resuspended in 400 µl of TES (10 mM TrisCl pH 7.5, 10 mM EDTA and 0.5% SDS) solution, 400 µl of phenol (H_2O buffered) and vortexed for 10 seconds. The samples were incubated at 65ºC for 60 minutes with the intermediate vortex of every 15 minutes. Afterwards, samples were shifted on ice for 5 minutes and centrifuged at 14000 rpm, 4ºC for 10 minutes. The aqueous layer was transferred to a new tube and 400 µl of chloroform was added. The sample was vortexed for 10 seconds and centrifuged at 14000 rpm, 4ºC for 10 minutes. Again the aqueous layer was transferred to a new tube. The RNA was precipitated by adding $1/10^{th}$ volume of 3 M NaOAc pH 5.2 and 2.5 volume of pre-chilled 100% alcohol and stored at -80ºC for 1 hr. RNA was precipitated by centrifugation at 14000 rpm, 4ºC for 10 minutes and the pellet was washed with 500 µl of 70% alcohol (with DEPC) at 14000 rpm, 4ºC for 10 minutes. Finally the pellet was resuspended in 50 µl of DEPC water. Concentration was determined by measuring at $OD_{260/280}$ (OD_{260} 1 means 40 µg/ml) and the quality of the RNA sample was checked by FA gel electrophoresis.

2.2.4 Yeast two-hybrid analysis:

Protein- Protein interaction studies were carried out in yeast two- hybrid strain Pj694-A. Bait vector plasmid pGBDUC1 harbored gene of interest which was fused to the binding domain and the target gene was cloned into prey vector having activation domain of the GAL4. Both the plasmids were transformed into the yeast strain and selected on double drop out plates Sc-Ura-Leu. The interaction was identified by selection on Sc-Ura-Leu-Ade or –His plates. Auto activation of the gene was confirmed by checking the interaction between either of the empty

vector of bait and prey vectors and the gene of interest. The Strong protein interaction was identified by observing growth on Sc-Ura-Leu-Ade plates, whereas weak protein interaction was identified by seeing the growth only in Sc-Ura-Leu-His plates.

2.3 Methods in biochemical experiments:

2.3.1 Recombinant protein expression and antibody generation:

PfalMRE11 ORF and different domains N- terminal, C-terminal domains were cloned individually into bacterial expression vectors pET 28 (a)+, pGEX6p2, pMAL and pET101/D. These constructs were individually transformed into bacterial expression strains BL21 (DE3), BL21 (DE3*), BL21 plys S, Codon plus and Rosetta. The transformed sample was directly used as primary inoculum and incubated in 37^{o}C incubator shaker at 200 rpm, for overnight. Secondary inoculum was given from overnight culture and grown until it reached OD_{600} 0.6-0.8. Recombinant protein expression was induced with 1 mM isopropyl thiogalactoside (IPTG, Sigma) and incubated for 4hrs in 37^{o}C shaker incubator at 200 rpm. Samples were collected from IPTG induced and un induced cultures. Protein induction was monitored on SDS-PAGE gel.

The induced protein band was used to raise the antibody in rabbit.

2.3.2 **Dot-blot analysis:**

Parasite genomic DNA was isolated from control and UV treated samples and stored in -20^{o}C. Probe (PfORC1 (1391 bp)) was PCR amplified and randomly labeled with [α-^{32}P] dATP using DecaLabel DNA labeling kit (Thermo scientific). The probe was prepared and stored at room temperature. DNA samples were prepared by mixing with 20x SSC (1.75 gm NaCl, 0.88 gm Na-citrate in 10 ml ddH_2o) solution and boiled for 5 mints. The samples were chilled on ice for 5 minutes and centrifuged at high speed. Nitrocellulose membrane and filter papers were presoaked in 10x SSC (350.67 gms NaCl, 176.47 gms sodium citrate dissolved for 4 L buffer). DNA samples were spotted on specified grids and allowed to dry. The membrane was

transferred to denaturation solution (32 gms NaOH, 140.28 gms NaCl dissolved in 4 L water) and incubated on pre-soaked filter papers for 30 minutes. Later the membrane was transferred to neutralization buffer (350.67 gms NaCl, 484.4 gms tris base, 286 ml HCL, 3714 ml water, pH 7.4) with filter papers and incubated for 30 minutes. The DNA sample was UV cross-linked at 15000 mJ. The membrane was then incubated with pre-hybridization buffer (300 ml of 10X SSC, 2.5 gms SDS, 0.5 gms Ficoll70, 0.5 gms Ficoll400, 1.0 gms polyvinyl phenol, 1.0 gms of BSA for 500 ml buffer) for 5 hrs at 65°C in hybridization chamber. The probe was PCR purified and boiled for 5 minutes and centrifuged before addition to the hybridization buffer (300 ml of 10X SSC, 25 gms SDS, 50 gms dextran sulfate for 500 ml buffer:). The membrane was incubated in hybridization buffer with radioactively labeled probe for overnight followed by washing with washing buffer for 1 hr at 55°C by changing the buffer every 15 minutes in hybridization chamber. The blot was finally incubated at room temperature for overnight and developed using phosphoimager.

2.4 Methods for DNA DSB repair assays:

2.4.1 MMS sensitivity assay:

Yeast strains BSB1-BSB8 and PVY1 were inoculated in synthetic complete media lacking tryptophan and incubated at 30°C incubator shaker, 200 rpm for overnight. Secondary inoculum was given and incubated at 30°C incubator shaker, 200 rpm until the OD_{600} reaches 0.8. The culture OD_{600} was normalized and serial dilution was done up to 10^{-4}. These samples were spotted on no MMS and MMS (0.005%, 0.01%) containing plates and incubated at 30°C incubator for 72 hrs, later each strain growth phenotype was observed.

The phenotype was quantified by dividing the secondary culture into two groups and one of them was treated with MMS and incubated at 30°C for 2 hrs. Later MMS was washed out and equal numbers of untreated and treated cells were spread on Sc-Trp plates and incubated at 30°C incubator. % survival of the cells was scored after 72 hrs.

2.4.2 Plasmid end-joining assay:

To perform NHEJ activity, the pRS413-kANMx6 plasmid was linearized with XhoI restriction enzyme. Approx 400 ng of the linearized and circular plasmid were transformed into the strains BSB1-BSB8 following yeast transformation protocol. NHEJ activity was measured depending on the efficiency of the recircularization of the plasmid. Transformants obtained were selected on agar plates containing G418 sulfate.

2.4.3 *In vitro* UV DNA damage sensitivity assay in *P. falciparum*:

P. falciparum 3D7 & Dd2 strain cultures were diluted to 1% parasitemia and divided into two groups. One of the groups was exposed to different doses of UV ranges from 50- 500 J/m^2. The post treatment culture was allowed to grow for 48 hrs in 37^{o}C parasite culture incubator and smears were made for untreated and treated culture. The % parasitemia was calculated by counting under the microscope.

2.4.4 DNA damage repair kinetics:

P. falciparum 3D7 culture with 2-3% parasitemia was divided into two groups and one of the groups was treated with 100 J/m^2 dose of UV. Untreated and treated cultures were allowed to grow for 48 hrs in 37^{o}C parasite incubator. Every 12 hrs parasite culture was harvested and genomic DNA was prepared following the standard protocol and untreated culture was harvested at the last time point. Genomic DNA concentration was normalized by dot blot analysis. Approx 10 ng of DNA was used to check the DNA damage recovery in the nucleus and mitochondrial genomes using organelle specific gene markers ORC1 and COX3 by real time PCR.

2.5 Methods in *P. falciparum* culture:

2.5.1 Washing blood RBC:

10 ml of blood was collected from a volunteer and 1.4 ml of CPDA was used as an anticoagulant. Blood serum was separated by centrifugation at 1500 rpm for 10 minutes at 4ºC. The serum layer (pale yellow colored) was aspirated with a Pasteur pipet attached to the vacuum pump. An equal volume of RPMI 1640 incomplete medium was added to the RBC left after aspiration and gently mixed. The whole sample was centrifuged at 2500 rpm for 10 minutes at 4ºC. The supernatant was aspirated and the washings were repeated twice. Finally equal volume of RPMI 1640 incomplete medium was added to the RBC pellet and mixed well. Washed RBC (50% hematocrit) was stored at 4ºC future use.

2.5.2 Method for thawing parasite from liquid nitrogen:

A vial of frozen parasite culture was thawed in pre-warmed Milli-Q water for 2-3 minutes. The vial was washed with 70% alcohol and transferred into a 50 ml centrifuge tube. For 1 ml of the parasite culture 0.2 ml of solution-I was added drop by drop/ sec and slowly the contents were mixed. The mix was incubated for 3-4 minutes in the laminar hood without disturbance. Later 10 ml of solution- II was added dropwise (1-2 drops/ sec) and mixed slowly by swirling the tube. Parasite culture was pelleted by centrifugation at 1000 rpm for 10 minutes and the supernatant was aspirated by Pasteur pipette. The pellet was loosened and 10 ml of solution-III per 1 ml of the blood was added dropwise (1-2 drops / sec). The sample was swirled to mix the contents. Parasite culture was centrifuged at 1000 rpm for 10 minutes and the supernatant was aspirated by Pasteur pipette. Finally, the pellet was resuspended in 3 ml of complete medium and transferred to the culture plate well having 5% hematocrit RBC.

2.5.3 Method for maintenance of parasite *in vitro* culture:

P. falciparum strains in vitro culture was done by following candle jar method. Parasite culture was maintained in RPMI 1640 complete medium with 1% Albumax II. 5% hematocrit RBC was used for maintenance and subculture. Every subculture was made at 3% parasitemia.

2.5.4 Method for parasite synchronization with sorbitol:

For synchronization, the culture with majority of the parasites being in ring stage was selected. 10 ml of the culture was pooled in a 15 ml falcon tube and was pellet down by centrifugation at 3000 rpm for 10 minutes at room temperature. Supernatant was aspirated and 1 ml of 5% sorbitol (preheated at 37°C) was added. The mix was incubated at 37°C water bath for 10 minutes and preheated incomplete medium was added up to 10 ml. The parasite culture was centrifuged at 3000 rpm for 10 minutes at room temperature. The pellet obtained was washed with RPMI 1640 incomplete medium 2-3 times. Finally, the parasite culture was resuspended in 10 ml RPMI 1640 complete medium and divided into two wells each with 5 ml.

2.5.5 Method for total RNA isolation from parasite culture (Trizol method):

Parasite culture was grown up to 10% parasitemia and then culture was treated with DNA damaging agents (0.05% MMS for 6 hrs and UV 100 J/m^2). 10 ml of the culture was harvested from treated and untreated parasite cultures for RNA isolation. The culture was harvested by centrifugation at 4°C, 1500 rpm for 10 minutes. The supernatant was aspirated through Pasteur pipette, and the pellet was washed with 45 ml of cold PBS. The pellet was then mixed with 25 ml of freshly made 1% acetic acid and incubated for 5 minutes on ice. The sample volume was brought to 45 ml with PBS and the parasites were pelleted by centrifugation at 3500 rpm for 10 minutes at 4°C. The supernatant was aspirated through Pasteur pipette. The parasite pellet was resuspended in 5 ml of Trizol (sigma) and mixed by vortex for 5 minutes followed by using syringing for 30 minutes (here parasites can be stored at -80°C for long time) until it

becomes homogenous. 1/5 volumes of $CHCl_3$ was added to the parasites and vortexed for 5 minutes at room temperature. The sample was then centrifuged for 30 minutes at 11000 rpm. The aqueous layer was transferred to a new tube and precipitated with the equal volume of isopropanol. The sample was incubated at -80ºC for 2 hrs. The sample was thawed on ice and centrifuged at 11000 rpm for 12 minutes. The supernatant was discarded and the pellet was mixed with 75% alcohol (RNase free). The RNA sample was transferred to 1.5 ml tubes and centrifuged at 12000 rpm for 5 minutes. The pellet was air dried and dissolved in 50 µl DEPC water. The sample was incubated at 55ºC for 10 minutes and stored at -80ºC. RNA integrity was analyzed by FA gel electrophoresis.

2.5.6 Method for parasite lysate preparation from asexual stages (for western blot analysis):

Parasite cultures were harvested and 2 volumes of 0.15% saponin (prepared with RPMI 1640 incomplete medium) was added to the packed cell volume followed by gentle mixing and incubation at 37ºC water bath for 15 minutes with intermittent vortexing. More than 5 volumes of RPMI 1640 incomplete medium was added to wash out the saponin. The parasites were collected by centrifugation at 4000 rpm for 10 minutes at 4ºC. The parasites were further washed 3-4 times with RPMI 1640 incomplete medium. The parasite pellet was resuspended in the Lammeli buffer for western blot analysis.

2.5.7 Method for total genomic DNA isolation from parasites:

Parasite infected erythrocytes were centrifuged at 3000 x g for 2 minutes. The pellet was washed with an equal volume of cold PBS. The cells were resuspended in 1 ml of cold PBS and transferred to a 2 ml tube. 10 ul of 5% saponin was added (for a final concentration of 0.05%) and inverted for several times until the solution turn to black. Parasites were centrifuged at 6000 x g for 5 minutes and the supernatant was removed. Parasites were mixed with 25 µl of lysis

buffer (40mM Tris-HCl, pH 8.0; 80 mM EDTA pH 8.0; 2% SDS and 0.1 mg/ml proteinase K – just before the reaction), 1 µl of proteinase K-20 mg/ml was added to it. The volume was finally brought to 100 µl by adding 75 µl of Milli-Q. The sample was incubated at 37°C for 3 hrs with intermittent stirring by hand (1 minute stirring for every 30 minutes). Later 300 µl of Milli-Q and 400 µl of PCIA (Phenol: Chloroform: Isoamyl alcohol) was added and mixed well. The sample was centrifuged at 12000 rpm for 15 minutes at room temperature. The aqueous layer was transferred to a new tube and 50 µl of RNase was added to remove RNA by incubation in 37°C water bath for 30 minutes. PCIA treatment was repeated as mentioned. Genomic DNA was precipitated by addition of 1/10 volume of sodium acetate and 2.5 volumes of absolute alcohol at -20°C for 2 hrs. The sample was centrifuged at 12000 rpm, 4°C for 30 minutes and the pellet was washed with 500 µl of 70% alcohol. The supernatant was discarded and the pellet was air dried. Finally, the sample was resuspended in 30 µl of Milli-Q (autoclaved).

2.6 Real-time PCR:

Total RNA was isolated from *P. falciparum* 3D7 strain blood stages and from the yeast strains BSB1-BSB8, PVY1. RNA concentration was measured by UV spectroscopy (JASCO spectrophotometer EMC-709). The sample was treated with DNase I (Fermentas) to remove DNA contamination and confirmed by negative PCR using genomic DNA primers (-RT PCR). cDNA was prepared using 10 µg RNA and reverse transcribed with reverse transcriptase enzyme (Qiagen). The cDNA quality was checked by semi- quantitative RT-PCR. Real time PCR was then performed using SYBR- Green kit (Roche & Takara) as per the manufacturer protocol and the reaction was run on Applied Biosystems 7500 Fast Real-time system. The threshold cycle (C_T) values of *ARP* or *PfPP1* was used to normalize the corresponding C_T values of *PfalMRE11* and other ORF's of putative DNA damage response genes transcripts. The relative levels of

mRNA were estimated from the formula ($=2^{\Delta CT}$). The mean values were plotted using Graph pad Prism 6 software.

2.7 Western blot analysis:

Western blotting was performed following standard protocol. Proteins were separated on SDS-PAGE and transferred onto (Poly Vinylidene di Fluoride) PVDF membrane by treating the membrane with methanol for 20 seconds, water for 2 minutes and 1x transfer buffer (5.86 gm glycine, 11.64 gm Tris base, 0.75 gm SDS, 400 ml methanol for 1 L 2X transfer buffer) for 5 minutes. The membrane was blocked with 5% blocking buffer (5 gm skimmed milk powder, 100 ml 1x TBS) for 2 hrs afterwards the blot was incubated with primary antibody overnight. Next day the blot was washed with TBST (0.2 M Tris base, 9% sodium chloride pH 7.6 and 0.1% Tween 20) for thrice. Secondary antibody conjugated with HRP was probed for 2 hrs and washed as previously described. The relative protein levels were visualized by using enhanced chemi-luminescence system (pierce). Band intensities were estimated by using the image J software. PfalMre11C recombinant protein expression was confirmed by using anti- His antibody (Santa Cruz Biotechnology Inc., CA) at 1:5000 dilution and HRP- conjugated anti-rabbit secondary antibody (Promega) at 1:1000 dilution was used. PfalMre11 protein levels from MMS treated and untreated were observed with the PfalMre11 antibody raised in rabbit. The antibody was used at 1:4000 dilutions and the secondary antibody HRP-conjugated anti- rabbit antibody (Promega) was used at 1:12000 dilution. The protein levels were normalized to PfHSP70 protein using anti- PfHSP70 raised in mouse at 1:1000 (kindly provided by Dr. Nirbhay Kumar, Tulane University) and the secondary antibody anti-mouse antibody (Promega) at 1:10000 dilutions.

2.8 Methods for bioinformatics analyses:

2.8.1 Multiple sequence alignment:

The Mre11 proteins sequence homology score was analyzed by online Expassy tool using Clustal W2 program. Evolutionary conserved Protein sequence residues were identified by DNA star tool.

2.8.2 Homology model:

PfalMre11 homology model was generated by submitting the full-length sequence to I-TESSAR (Iterative Threading ASSEmbly Refinement) web server. The server used 4FBK and 3T1I PDB protein structures as templates to generate the model to the nuclease domain of Mre11. The unaligned sequences were remodeled by using Ab-Initio Modeling. The homology model for nuclease domain was generated with highest confidence. To visualize the model images, PyMOL molecular visualization tool was used.

2.8.3 Psipred tool to predict the secondary structure of protein:

PfalMre11 and ScMre11 protein sequences were submitted to online server psipred tool. Secondary structures of these two proteins were analyzed and at coiled- coil regions and were selected to construct the chimera proteins. Accordingly the primers were designed to the corresponding DNA sequence to generate chimeras used in Mre11 complementation studies.

Table 2: List of Yeast Strains

Strains	Genotype
W303α	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1*
MKB7	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158)*
BSB1	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA*
BSB2	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ ScMRE11*
BSB3	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ PfalMRE11*
BSB4	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ chimera1*
BSB5	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ chimera2*
BSB6	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ ScMRE11C*
BSB7	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ ⊿343chimera1*
pJ694-A	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ*
BSB14	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1/ PfalMRE11*
BSB17	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1/ yXRS2*

BSB19	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1/PfalMRE11, pGADC1/ PfalMRE11*
BSB20	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1*
BSB22	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1/ yXRS2, pGADC1/ chimera1*
BSB23	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1, pGADC1*
BSB24	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA, LYSZ :: GALJ-HIS3 GAL2-ADE2 metZ::GAL7-lacZ, pGBDUC1/ yXRS2, pGADC1/ ScMRE11*
PVY1	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/ Chimera 1 (D398N)*
PVY2	*MATa leu2-3, 112 his3-11, 15 ade2-1, trp1, ura3-1, mre11⊿::URA3, VIIL::ADE2 (pSD160), LEU2 (pSD158), pTA/mre11(D56N)*
BSB25	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALJ-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ ScXRS2, pGADC1/ PfalMRE11*
BSB26	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALJ-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ ScXRS2, pGADC1/ chimera 1*
BSB28	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALJ-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfalRAD50 pGADC1*
BSB30	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALJ-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfalRAD50, pGADC1/PfalMRE11*
BSB32	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALJ-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/PfalMRE11, pGADC1/ScMRE11*
BSB33	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALJ-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ScMRE11 pGADC1/ScMRE11*

SAN1	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ScRAD50*
SAN2	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ ScRAD50, pGADC1*
SAN3	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ ScRAD50, pGADC1/ PfalMRE11*
SAN4	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ ScRAD50, pGADC1/ ScMRE11*
SAN5	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/ ScRAD50; pGADC1/ chimera 1*
SAN7	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/YKU80 pGADC1*
SAN8	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/YKU80, pGADC1/ScMRE11*
SAN9	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/YKU80, pGADC1/PfalMRE11*
SAN10	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14Δ ga180Δ LYS2 :: GALl-HIS3 GAL2-ADE2, met2::GAL7-lacZ pGBDUC1/YKU80 pGADC1/ chimera 1*

Table 3. List of Bacterial strains

Strain	Genotype
Top 10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
Rosetta	F^- ompT hsdS_B(R_B^- m_B^-) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)

Table 4: List of Primers

Primer	Sequence (5' to 3')	Purpose
OMKB023 (FP)	ATCCGGATCCATGAAAAGTACACAAAGTAATTTG	To amplify PfalMre11 to clone in pTA
OMKB024 (RP)	ATCCGGATCCTTATTTTTTTTCCTATGAGGTG	To amplify PfalMre11 to clone in pTA
OMKB084 (FP)	GGATCCATGGACTATCCTGATCCAGAC	To amplify ScMre11 to clone in pTA
OMKB085 (RP)	CTGCAGCTATTTTCTTTTCTTAGCAAGGAGAC	To amplify ScMre11 to clone in pTA
OMKB089 (RP)	CTGCAGTTATTTTTTTTCCTATGAGGTG	To amplify PfalMre11 C terminal region for chimera 2
OMKB098 (FP)	AAGCTTCATTTGAGGCCTCACGATAAAGATG	To amplify ScMre11 C- terminal for chimera 1
OMKB099 (FP)	GGATCCATGAGTAAAAATGATGCAAGTAC	To amplify PfalMre11 nuclease domain for (Δ343aa)chimera 1
OMKB100 (RP)	AAGCTTATAAGGTTTAGAAAAATGTTC	To amplify PfalMre11 N- terminal for chimera 1
OMKB129 (RP)	AAGCTTTTAATAAGGTTTAGAAAAATGTTC	To amplify N-terminal domain of PfalMre11 sequence
OMKB153 (FP)	AAGCTTTCTAAACCTTATTTAGAAGAAAG	To amplify PfalMre11 C- terminal for chimera 2
OMKB154 (RP)	AAGCTTATTTTTAATTGGATTGTGTAC	To amplify ScMre11 N- terminal for chimera 2
OMKB161 (FP)	GGATCCATGCATTTGAGGCCTCACGATAAAGATG	To amplify ScMre11 C- terminal for ScMRE11C construct
OMKB166 (RP)	GTCGACTTATTTTTTTTCCTATGAGGTG	To amplify PfalMre11 C terminal sequence for chimera 2
OMKB167 (FP)	GAATTCATGACTACGCTTGAAAAGATTG	To amplify PfalRad50 fragment-1
OMKB188 (FP)	GGATCCATGAAAGATATAAAACTAGCTG	To amplify PfalMre11 C terminal for chimera 2
OMKB254 (FP)	TTCGATGATGAAGATACC	Sequencing primer for pGADC1 vector
OMKB255 (RP)	TGAAGTGAACTTGCGGGG	Sequencing primer for pGADC1 vecror
OMKB299 (FP)	ATTAAACAGTGGAAATTTATTTC	To create D398N mutation in chimera 1
OMKB298 (RP)	TTTATTTTTATGAAATAAATTTCC	To create D398N mutation in chimera 1
OMKB326 (RP)	CTGCAGTCAAGTATTAACTCTTTCAATTTTTG	To amplify PfalRad50 fragment-2
OMKB327 (RP)	TTAATACGAATGAAAGATAAGAAC	To amplify PfalRad50 fragment-1
OMKB328 (FP)	TATGAAGATGATGATCATCATTC	To amplify PfalRad50 fragment-2
OMKB175 (FP)	AGATGAAGATGAAGGAGAC	To check expression levels of PfalMre11 and chimera 2

OMKB077 (FP)	TTTTCTTTTCTTAGCAAGGAG	To check expression levels of ScMre11, chimera 1, ScMRE11C and D398N genes
OMKB329 (RP)	GAACCTACTTCAACTTGTATCC	To check the PfSufB expression
OMKB330 (FP)	AATCCACAGAGAAATTACAAAATC	To check PfEXO1 expression
OMKB331 (RP)	TATGATCAATGTAAGGATTACAATG	To check PfEXO1 expression
OMKB332 (FP)	ATTCGAGATAAAGGAATTATTGAAG	To check PfSgs1 expression
OMKB333 (RP)	ATGCTTCCTCTTTACATTCATAG	To check PfSgs1 expression
OMKB334 (FP)	ATGAACATAGTGTTAATAATATGC	To check PfWRN expression
OMKB335 (RP)	TATAAACGAACTGATCTAATATATC	To check PfWRN expression
OMKB336 (FP)	TTAAGTGACAGTAGACCAATCG	To check PfPCNA1 expression
OMKB337 (RP)	ATCTTTATTATCCATATCGTCATC	To check PfPCNA1 expression
OMKB338 (FP)	AAGATATCTAGTCATGTTCTCAAG	To check PfPCNA2 expression
OMKB339 (RP)	AATGAGATGGATCTTGTAGGTC	To check PfPCNA2 expression
OMKB340 (FP)	TAAACAAGATATTATTGAAGAAAGG	To check PfSrs2 expression
OMKB341 (RP)	TTTCATTAAACCTCGAAACAG	To check PfSrs2 expression
OMKB342 (FP)	AATGTAACAAATTGGAATGCAATC	To check PfRAD1 expression
OMKB343 (RP)	TTCTAATTGCTCTAATGTTTGTTC	To check PfRAD1 expression
OMKB344 (FP)	TACAACAGATTGTATAACACTTAC	To check PfERCC1 expression
OMKB345 (RP)	TTAAATGTAGCCATTAATGCCTG	To check PfERCC1 expression
OMKB346 (FP)	AATTCACCTCTAAAGGAAGATTC	To check PfFEN1 expression
OMKB347 (RP)	TACATTAGTAACATCCTTTGGAC	To check PfFEN1 expression
OMKB348 (FP)	ATATGCAGATGATAAAGGTATTGG	To check PfCDC9 expression
OMKB349 (RP)	TTATTACTAAATTGTGCCTCA	To check PfCDC9 expression
OSB050 (RP)	CAAACGAGTGCGAACTGCAAC	To check expression levels of ScMre11, chimera 1, ScMRE11C & and D398N
OSB014 (FP)	TTAGAAACACTTGTGGTGAACG	To check expression levels of actin
OSB016 (RP)	TGACCAAACTACTTACAACTCC	To check expression levels of actin

OSB094 (FP)	CTGTAACACATAATAGATCCGAC	To check expression levels of ARP in *P. falciparum* 3D7
OSB095 (RP)	TTAACCATCGTTATCATCATTATTTC	To check expression levels of ARP in *P. falciparum* 3D7
OSB139 (FP)	CAAATGTAACTTCTGATTTTACATC	To check expression levels of PfalMre11 in *P. falciparum* 3D7
OSB140 (RP)	AACATGTGAACAGCTACAATATG	To check expression levels of PfalMre11 in *P. falciparum* 3D7
OMKB262 (FP)	GGATCCATGTGGGTAGTACGATACCAG	To amplify yXRS2 from yeast genomic DNA
OMKB263 (RP)	GGATCCTTATCCTTTTCTTCTTTTGAA	To amplify yxrs2 from yeast genomic DNA
OMKB164 (FP)	GGATCCATGAGCGCTATCTATAAATTATC	To amplify ScRad50 from yeast genomic DNA
OMKB165 (RP)	GAATTCTCAATAAGTGACTCTGTTAATATC	To amplify ScRad50 from yeast genomic DNA

Table 5: List of Plasmids

S. No	Plasmid	Purpose
1	pTZ57R/T	TA cloning vector for intermediate cloning the gene of interest
2	pTA	Yeast expression vector with bidirectional promoter having TRP as selectable marker in yeast and ampicillin in bacteria
3	pGBDUC1	Bait vector in yeast-two hybrid system
4	pGADC1	Prey vector used in yeast-two hybrid system
5	pET28 A (+)	Bacterial expression vector

RESULTS

3.1 DNA damage sensitivity of *P. falciparum* strains of different geographical origin:

DNA repair systems are crucial for protecting the genome stability of an organism. Bioinformatics analysis of *P. falciparum* genome reveals the divergence of evolutionarily conserved DNA repair proteins in this parasite. Many of the DNA repair proteins are not identified via *in silco* analysis suggesting their apparent absence in *Plasmodium*. *In vitro* studies on repair of UV mediated DNA damage with cell lysates from different *P. falciparum* strains exhibited differential repair efficiency of *UV* DNA lesions (Trotta et al. 2004), implying defective DNA repair system in drug resistant malaria parasites (Bethke et al., 2007). In order to investigate the DNA damage sensitivity of different *P. falciparum* strains we have analyzed two different *P. falciparum* strains, namely 3D7 and Dd2, originated from two different parts of the world. Additionally, 3D7 strain is sensitive to antimalarial drug chloroquine and Dd2 strain is chloroquine resistant. When exposed to different doses of UV, both the strains were found to be similarly sensitive to UV mediated DNA damage. Return-to-growth experiment revealed that both the strains could repair DNA damage caused by even higher dose of UV radiation, suggesting that *P. falciparum* strains have active DNA repair mechanisms (Figure 9). Further, we have compared the DNA damage sensitivity of *P. falciparum* strains with unicellular eukaryotic model organism *S. cerevisiae* wild-type strain (w3o3) and ⊿*rad51* strain. As expected the DNA repair defective strain ⊿*rad51* is highly sensitive to DNA damage compared to the DNA repair proficient strain W3o3. Surprisingly, both of the *P. falciparum* strain exhibited marked difference in UV sensitivity than the W303 yeast strain. The *P. falciparum* strains were less sensitive to UV than yeast (Figure 9). These data suggest that *P. falciparum* has evolved with more efficient DNA repair mechanisms.

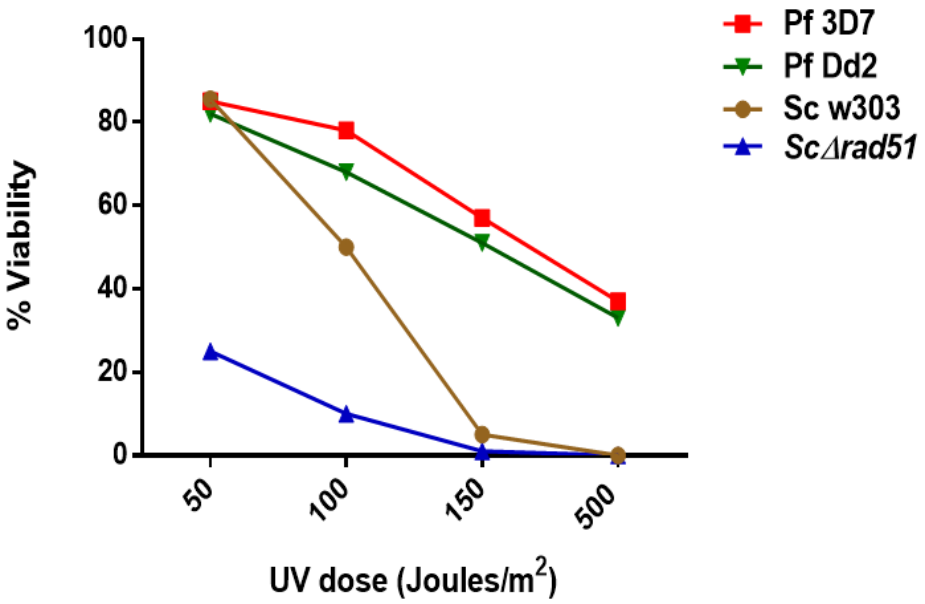


Figure 9: UV DNA damage sensitivity of different strains of *P. falciparum*. Line diagram represents cell survivability of the *Plasmodium* strains and *S. cerevisiae* wild-type and deletion mutant strains at different doses of UV mediated DNA damage. Y-axis indicates % survivability and the X- axis indicates the cell survivability at different UV doses (J/m^2).

3.2 *P. falciparum* DNA repair mechanisms are active during all the stages of intra-erythrocytic developmental:

P. falciparum exists in three different developmental forms during its erythrocyte life cycle: ring, trophozoite and schizont stages (Figure 10 A). Previously DNA damage repair studies carried out with DNA damage agent MMS showed that parasite could repair DNA damage (Gopalakrishnan et al. 2013 and Bhattacharyya et al. 2003). In order to investigate whether all the three stages of the parasites are equally efficient in DNA repair we performed the following experiment. We have treated parasite cultures synchronized at any of the three developmental stages with different doses of UV radiation and allowed the culture to grow for one generation. We calculated the percent parasitemia of untreated and treated cultures after one generation (48 hrs). We found that all the three cultures could repair DNA damage and exhibited similar parasitemia (Figure 10 B). This result suggest that *P. falciparum* DNA damage repair mechanisms have the ability to repair DNA damage created by UV and are active at all the three developmental stages. Next we sought to determine the repair kinetics of the nuclear as well as the mitochondrial genome. The repair kinetics assay employed to find out the minimum time required to repair the damaged created mitochondrial or nuclear genome is outlined in Figure 11. We found that *P. falciparum* mitochondrial and nuclear genome DNA were repaired between 12 hrs to 24 hrs (Figure 12 A & B).

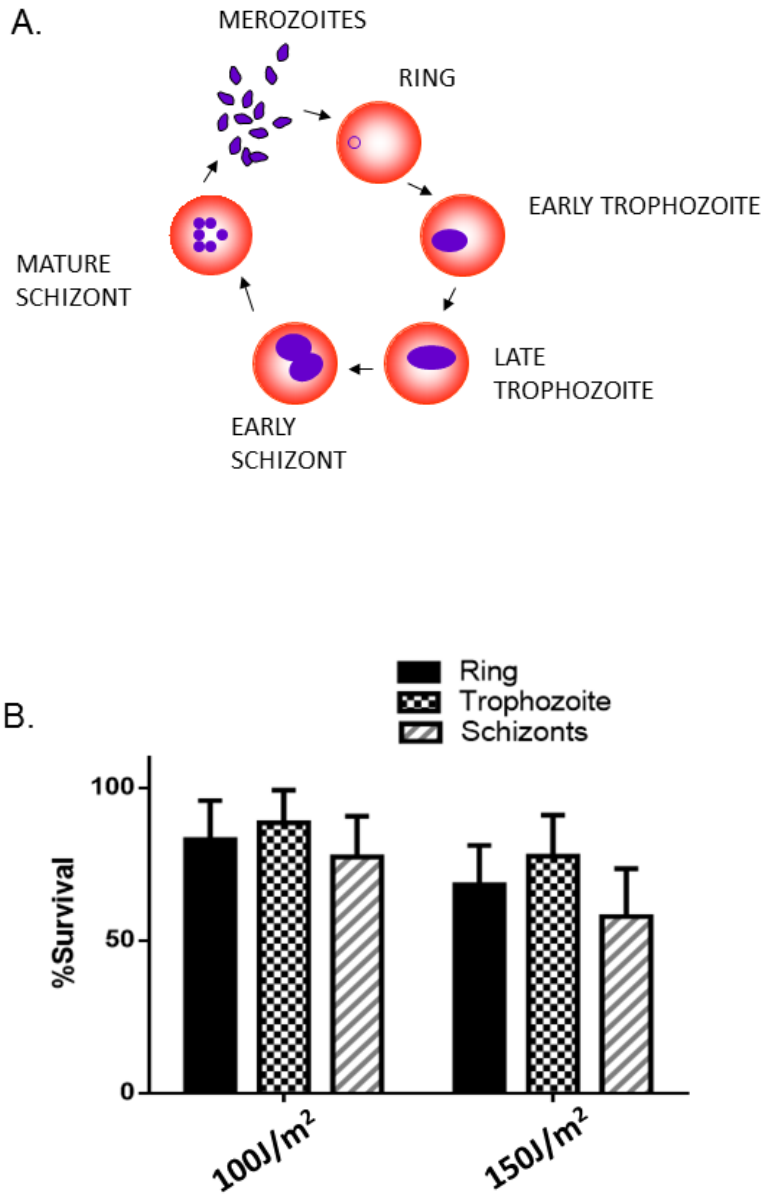


Figure 10: UV DNA damage sensitivity during intra-erythrocytic growth of *P. falciparum.* A) Intra-erythrocytic developmental forms of *P. falciparum.* B) Bar diagram representing parasite survivability upon UV DNA damage. X- axis indicates the UV dose and Y- axis indicates the % parasitemia compared to un-treated culture synchronized at ring, trophozoite and schizont stages.

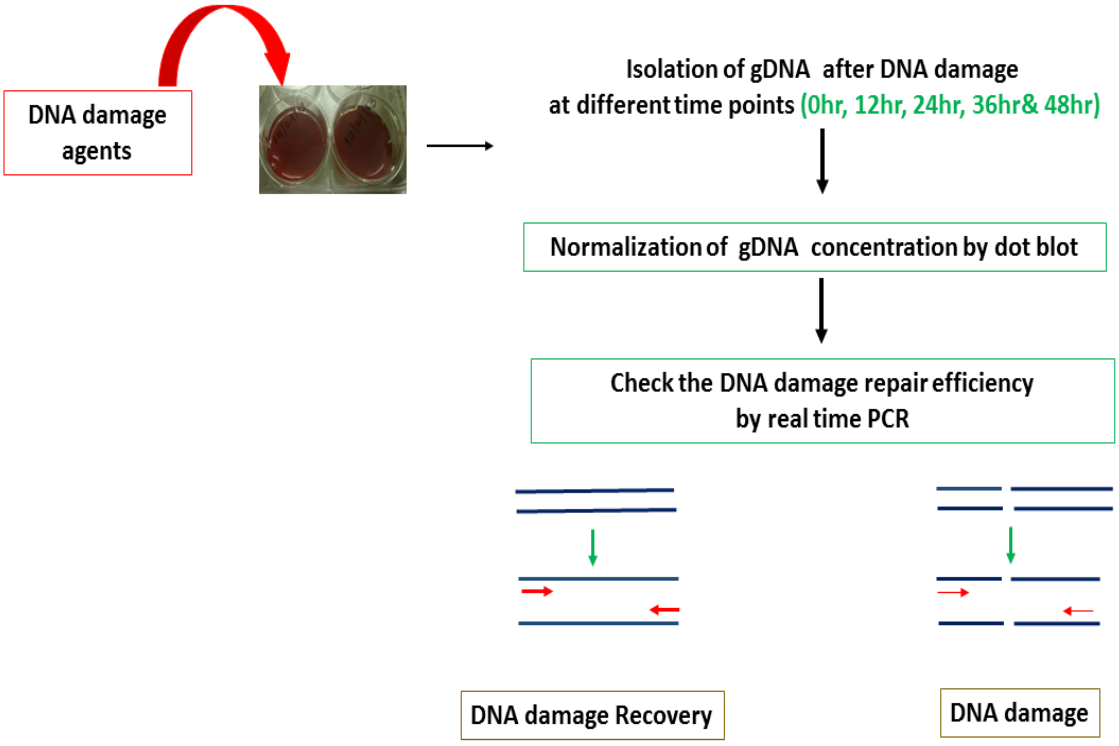


Figure 11: Schematic representation of the assay employed for measuring kinetics of DNA repair post MMS treatment in *P. falciparum*.

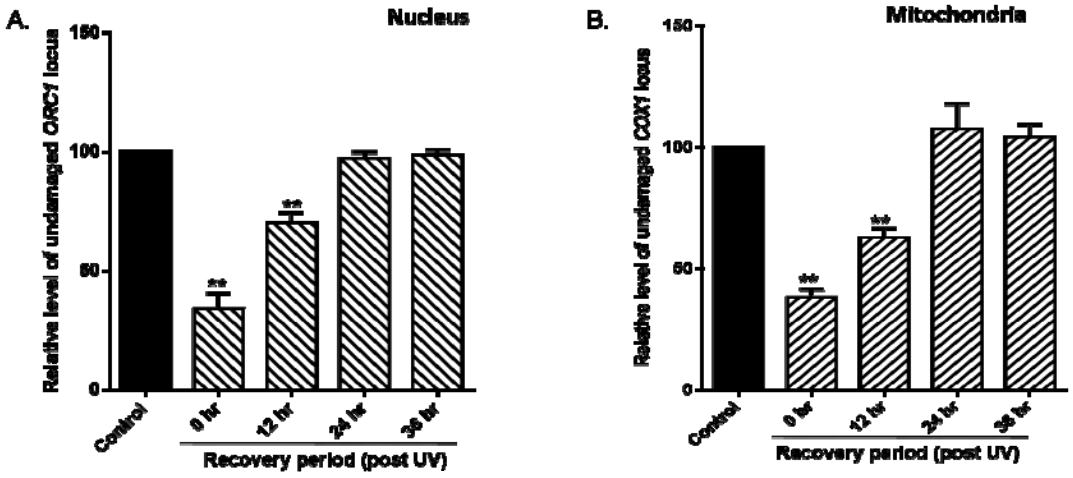


Figure 12: Kinetics of DNA damage repair of nuclear and mitochondrial genome. A) Time kinetics of recovery of nuclear genome as measured at the *ORC1* locus. B) Time kinetics of recovery of mitochondrial genome as measured at the *COX1* locus.

3.3 Putative DNA repair genes of *P. falciparum* are up-regulated in response to DNA damage:

DNA repair mechanisms are poorly understood in protozoan parasite *P. falciparum*. In model organisms it has been found that a DNA DSB is sensed by sensor proteins and then this information is transduced by a cascade of transducer kinases, which eventually activate and recruit components of the effector mechanisms to mend the DSB (Figure 13). In *P. falciparum*, proteins involved in the effector mechanism (especially molecular players belonging to the HR pathway) have been identified. The signaling kinases or the sensor proteins (except for PfalMre11) have remained illusive in this parasite.

A few of the DSB repair genes of *P. falciparum* have been identified and characterized. A recent genome mining approach has also identified several putative orthologs of important genes in Plasmodium genome data base (Lee et al. 2014). In an attempt to investigate whether the DSB repair pathway of *P. falciparum* is activated in response to DNA damage, we have selected 13 genes belonging to this pathway and compared their transcriptional profile before and after DNA damage. We found that most of the genes are up-regulated upon MMS mediated DNA damage (Figure 14). The DNA damage response sensor protein PfalMre11 was found to be up-regulated. We also observed up-regulation of helicases, nucleases, recombinases and DNA ligase. These data collectively suggest that the putative orthologues of DNA repair genes are likely to have key functional role in DSBs repair (Figure 14 & 15).

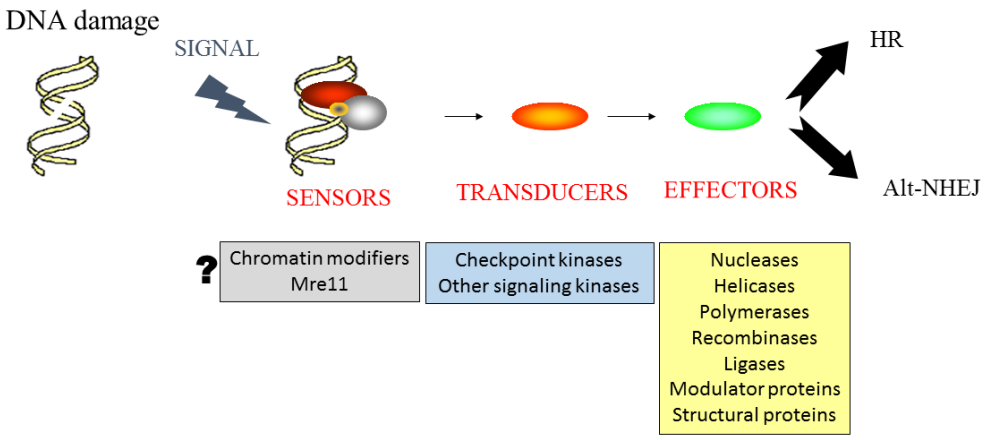


Figure 13: Schematic representation of proposed DNA damage response pathway in *P. falciparum.* The sensor proteins are not well defined in this parasite (as marked by a question mark). The transducers are also not identified in *P. falciparum* genome.

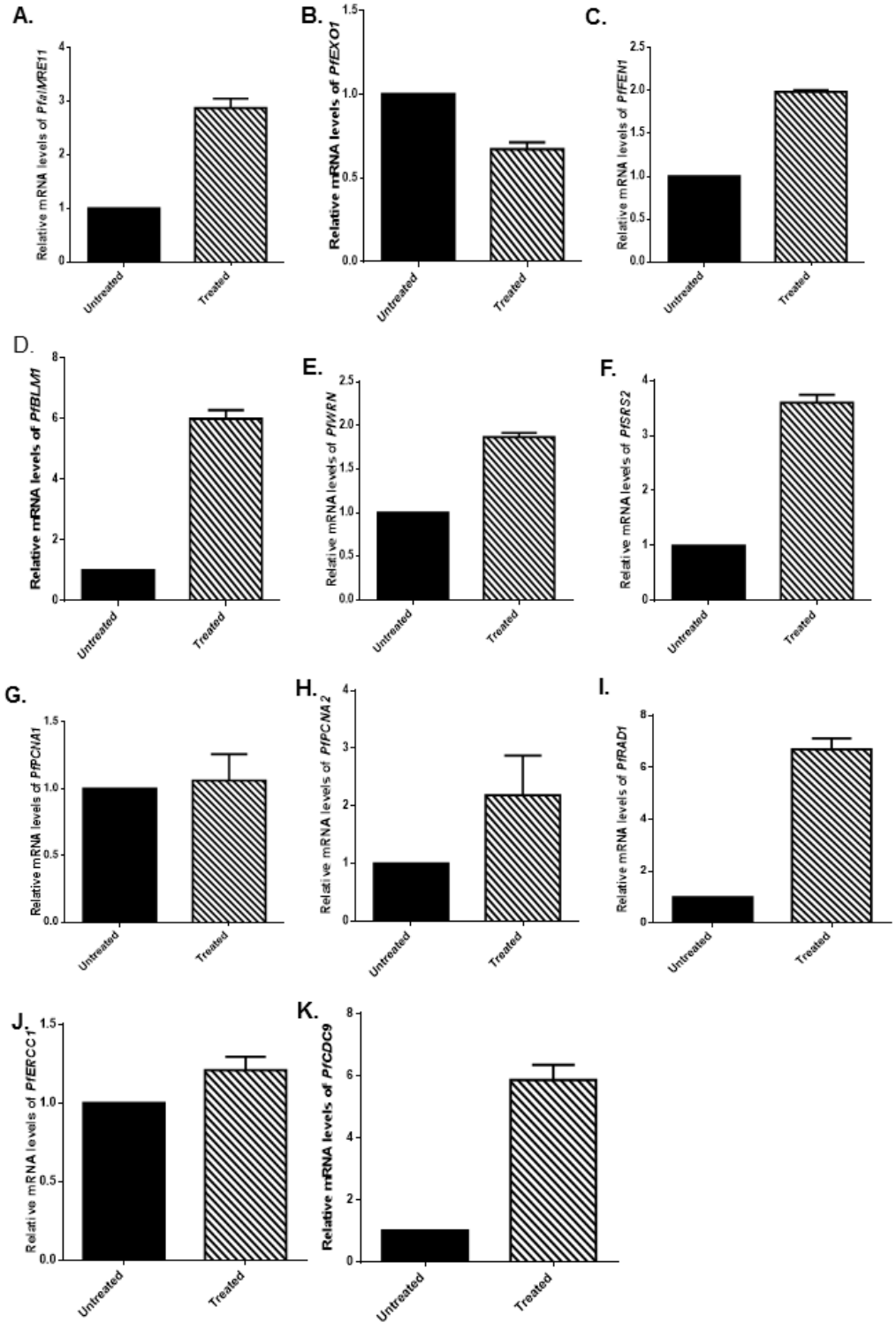


Figure 14: Transcript analysis of selected DNA repair genes upon DNA damage induction. A (*PfalMRE11*), C (*PfFEN1*), D (*PfBLM1*), E (*PfWRN*), F (*PfSRS2*), H *(PCNA2*), I (*PfRAD1*), J (*PfERCC1*) &H (*PfCDC9*) represents the up regulation of the putative DNA repair genes in *P. falciparum*. B (*PfEXO1*) & G (*PfPCNA1*) show the down regulation or no response of putative genes upon DNA damage.

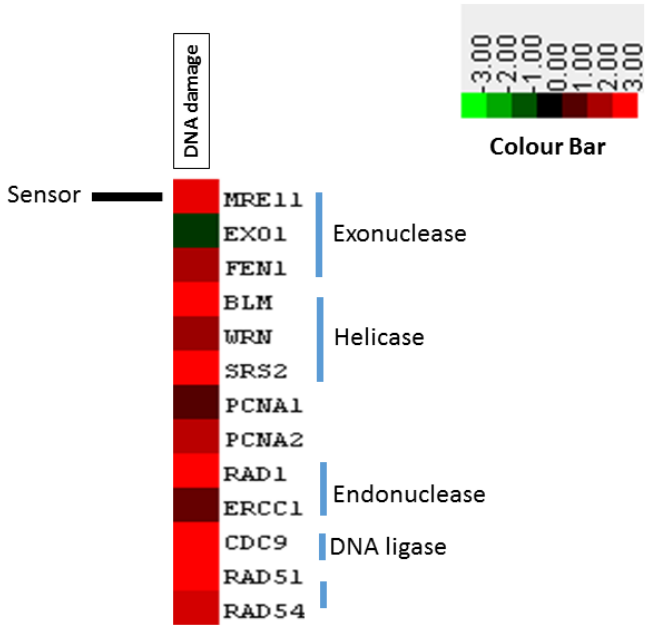


Figure 15: Heat map generated for genes involved DNA damage response upon MMS treatment in *P. falciparum.* Putative orthologues of DNA damage response genes of *P. falciparum* are marked. Red represents transcriptional up-regulation; green represents down-regulation and black represents no change at transcript.

3.4 Primary structure analysis of Mre11 orthologue in *P. falciparum*:

A BLAST search performed in PlasmoDB using *S. cerevisiae* Mre11 as a template revealed a single copy of putative Mre11 ORF (Gene ID: PF3D7_0107800). The ORF coding sequence has single exon with 3,699 bp (designated as PfalMre11). The ORF was PCR amplified from genomic DNA and cloned into TOPO Blunt vector and confirmed by DNA sequencing. PfalMre11 is encoded by 1233 amino acids with a molecular mass of 146 kDa and the isoelectric point of 5.35. Homology search across the Mre11 orthologs in model organisms revealed 14%-22% similarity. However, nuclease domain showed considerable homology of 33%-35% to the nuclease domains of model organisms (Table 6). Evolutionarily the average length of the Mre11 orthologs are around 700 amino acids, PfalMre11 has the longest ORF with 1233 amino acids. A genome database search in apicomplexan protozoan revealed a putative ortholog in *Toxoplasma gondii* (close apicomplexan parasite) which is similar to that of PfalMre11 (encodes for 1244 amino acids).

Evolutionarily Mre11 orthologs contain four highly conserved phosphodiesterase motifs and DNA binding domain. All the four phosphodiesterase motifs are present in the N- terminal nuclease domain. Interestingly *E.coli* Mre11 ortholog EcsbcD contain the first three motifs only. Unlike the other Mre11 orthologs, PfalMre11 has a unique N-terminal extension just before the nuclease domain (351-796aa) encoding for 350 amino acids (Figure 16 A). A similar observation is seen in *T. gondii* TgMre11 which has 220 amino acids N-terminal extension preceding its nuclease domain. Such a long N-terminal extension is found only in these two apicomplexan parasites. Mre11 functions are well characterized in yeast, human and other model organisms. In yeast, Mre11 nuclease functions are attributed to four phosphodiesterase motifs. Amino acids critical for nuclease function are characterized (Moreau et al. 1999) in these motifs, they are D16, D56, H125, and H213. These amino acid residues are evolutionarily conserved and respective amino acids are present in PfalMre11 at D358, D398, H706, and H792 positions (Figure 16 B & C). The spacing between the Phosphodiesterase motifs is tentatively conserved among the all

Mre11 orthologs. The spacing between two motifs rages from 30-40 aa (between motif 1 and 2), 69-76 aa (between 2nd and 3rd), 87-99 aa (between 3rd and 4th). In PfalMre11, this type of spacing pattern is conserved only between 1st -2 nd and between 3rd -4th motifs. There is a large low complexity region between 2nd and 3rd motifs with 308 amino acids with unknown function. This kind of insertion is not observed in any parasite Mre11 orthologues like TgMre11 or TbMre11.

Yeast Mre11 contains two DNA-binding domains (DBD), one is called DBD-A which is rich in basic amino acids and the other one is DBD-B rich in acidic amino acids (Usui et al. 1998, Furuse et al. 1998). Mutational studies in yeast showed that DBD-A is essential for Mre11 functional activities, whereas DBD-B is dispensable for Mre11 functions (Furuse et al. 1998). Mre11 domain structure analysis showed that PfalMre11 is consistent with DBD-A and DBD-B is absent. Similar observation also was seen in TgMre11.

Studies carried out with yeast and mammalian Mre11 proteins reported that Mre11 C-terminal domain plays crucial role during DDR signaling (Bhattacharyya et al. 2008) even though the C-terminal Mre11 sequence shows poor homology across the Mre11 orthologs. In case of PfalMre11, it is difficult to predict the presence of DDR signaling function due to lack of homology. A470 motif present in the α- helical region with thirteen amino acids was found to be conserved in all the eukaryote Mre11 orthologues except in dipterans. In Yeast, its function was characterized by showing it regulatory role in telomere recombination and in telomere rapid deletion (Joseph et al. 2010). Surprisingly this motif seems to be absent in *Plasmodium* Mre11 but present in other protozoan TgMre11 and TbMre11.

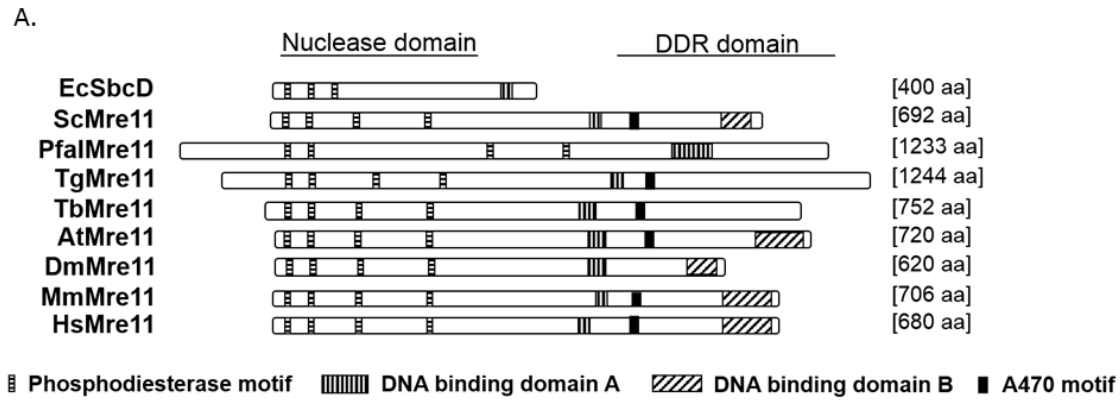
We have compared the N-terminal regions of PfalMre11 and ScMre11 with the help of three-dimensional structures, which revealed that the core nuclease domains showed near perfect superimposition despite having low homology sequence (32%). The three-dimensional superimposition model revealed that the N-terminal extension (region I) and low complexity

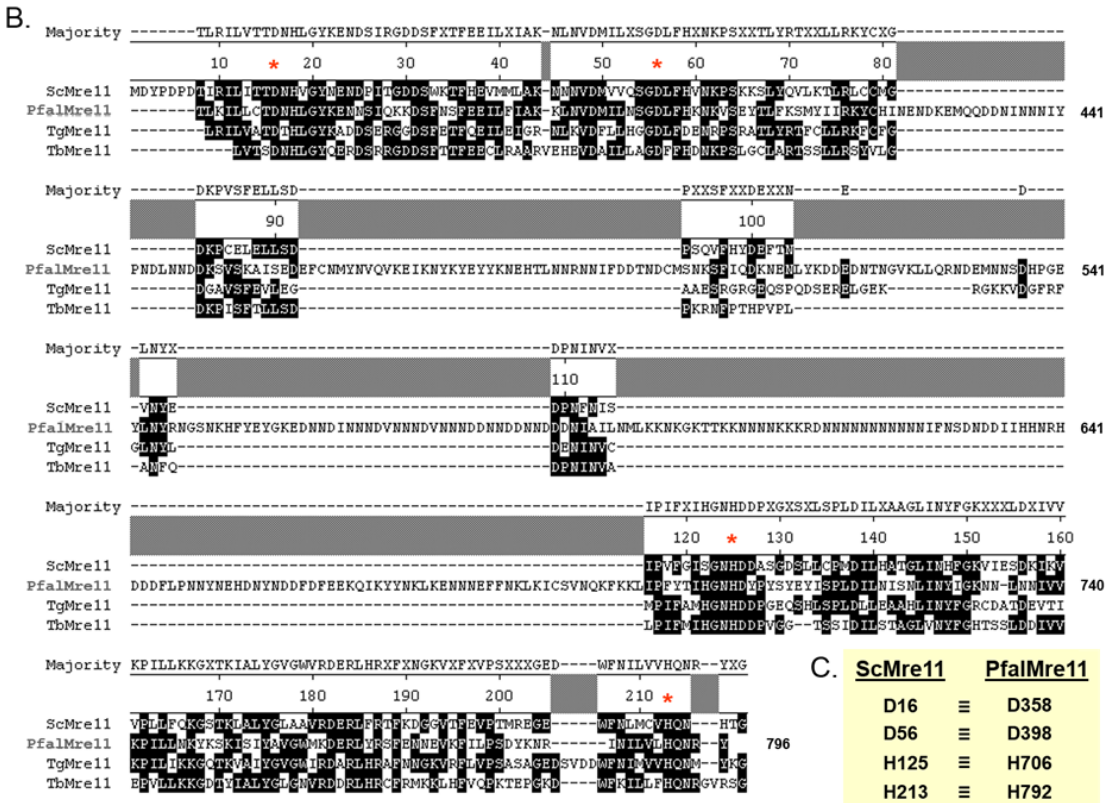
region found in 2nd -3rd phosphodiesterase motif (region II) protrude out of the core structure without any change in protein tertiary structure (Figure 16 D).

Table 6: Homology score of PfalMre11 with other eukaryote Mre11 orthologs

	ScMre11	PfalMre11	TgMre11	TbMre11	AtMre11	DmMre11	MmMre11	HsMre11
ScMre11	100	23 (32)	31 (41)	26 (43)	30 (45)	32 (44)	34 (49)	34 (50)
PfalMre11		**100**	**14 (34)**	**17 (35)**	**22 (34)**	**23 (34)**	**21 (36)**	**22 (37)**
TgMre11			100	27 (45)	32 (42)	31 (43)	35 (47)	35 (46)
TbMre11				100	29 (49)	30 (49)	29 (49)	30 (50)
AtMre11					100	36 (50)	35 (56)	36 (56)
DmMre11						100	41 (57)	39 (56)
MmMre11							100	89 (96)
HsMre11								100

Sc- *S. cerevisiae*; Pfal- *P. falciparum*; Tg- *T. gondii*; Tb- *T. brucei*; At- *A. thaliana*; Dm- *D. melanogaster*; Mm- *M. musculus*; Hs- *H. sapiens*. Numbers in perantesis indicates the homology score within the nuclease domain





C.

ScMre11		PfalMre11
D16	≡	D358
D56	≡	D398
H125	≡	H706
H213	≡	H792

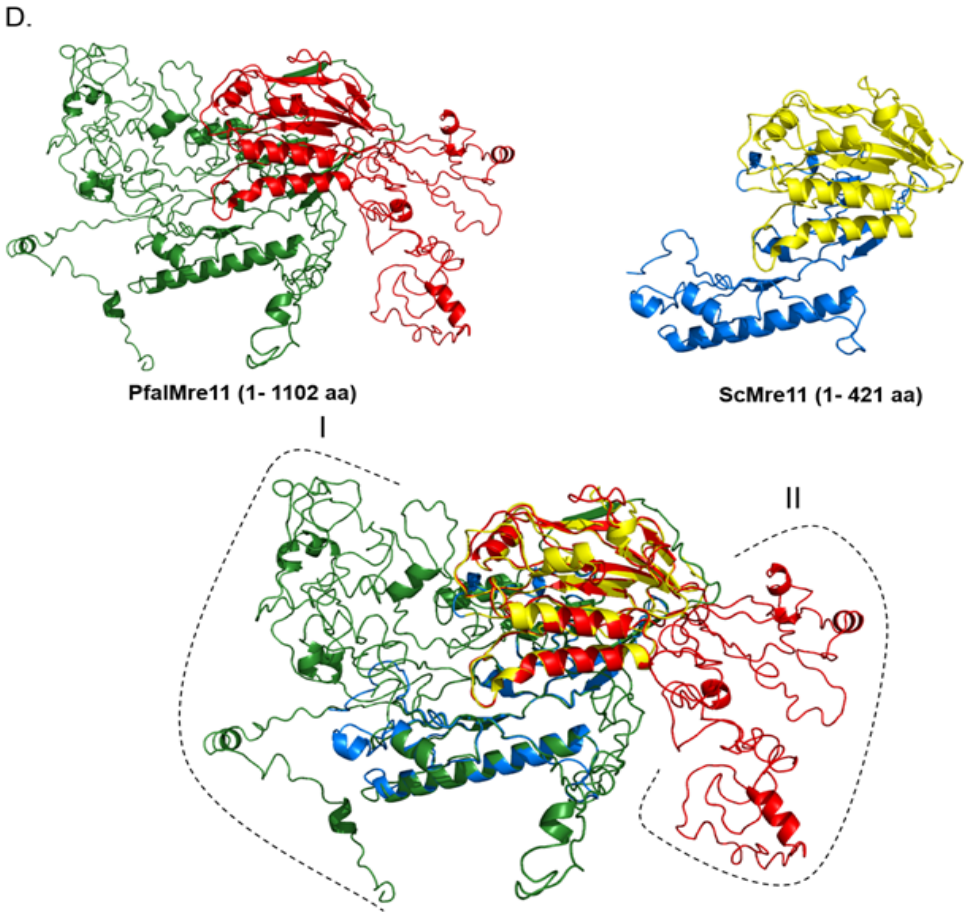


Figure 16: Primary structure analysis of PfalMre11. A. Illustration of different domains and motifs present in PfalMre11 with respect to other eukaryotes (*Saccharomyces cerevisiae, Toxoplasma gondii, Trypanosoma brucei, Arabidopsis thaliana, Drasophila melanogaster, Mus musculu and Homo sapiens*) Mre11 orthologs and prokaryote ortholog *E. coli* SbcD. Four of the nuclease domains are conserved in PfalMre11, but not the C-terminus DDR domain. PfalMre11 has unique extinction sequence at the N-terminus. DNA binding domain B and A470 motif (AVNN-NN(E/K)FV(E/D)K(D/E)(D/E)KNN-NNA, where NN-NN refers to sites that accommodate multiple residues). **B.** Multiple sequence alignment showing sequence similarity within the nuclease domain of PfalMre11 corresponding to other Mre11 orthologs, TbMre11 and TgMre11 represents the protozoan parsites. Sequence identity is highlighted with back shades. Nuclease function critical residues in each phosphodiesterase motif (D358, D398, H706 and H792) are marked with red asterisk. The amino acid positions are marked according to ScMre11 amino acid sequence (on top) and PfalMre11 amino acid position is presented on right. **C.** The critical amino acid positions in each phosphodiesterase motif of PfalMre11 are represented in correspondence with ScMre11 (D16, D56, H125 and H213). **D.** Homology model of PfalMre11 N-terminus domain. Homology models for PfalMre11 (1-1102aa) and ScMre11 (1-421aa) are shown. The nuclease domain of PfalMre11 is represented with red and rest of the sequence with green, while the nuclease domain of ScMre11 is represented with yellow and the rest of the sequence with marine blue. The long N-terminus extension as region I and several insertion sequences in nuclease domain as region II are also shown.

3.5 PfalMre11 recombinant protein expression and generation of antibody:

The PfalMRE11 ORF was cloned into various bacterial expression vectors (pET101/D, pET28a (+), pGEX-6p2 and pMAL-c2). The recombinant protein expression was monitored by IPTG induction in different bacterial expression strains of *E. coli* (BL21 DE3, BL21 DE3*, Rosetta DE3, BL21 pLys S and BL21 codon plus). Western blot analysis using anti-His antibody and other tag specific antibodies confirmed that the recombinant protein was not expressed. The PfalMre11 N-terminus (832aa) and the PfalMre11 C-terminus (377aa) domains were separately cloned into bacterial expression vectors (mentioned above) and transformed into bacterial expression strains. The PfalMre11 C-terminus cloned into pET28 a (+) expression was observed in *E. coli* strain Rosetta DE3 strain (having pRARE plasmid that supplies t-RNAs for six rare amino acids) with 1mM IPTG induction (Figure 17 A). Recombinant protein expression was confirmed by western blot analysis using ant-His antibody (Figure 17 B). The recombinant protein expression was estimated to be 15% in the total cellular protein and found to be present in insoluble fraction (confirmed by western blot and SDS PAGE). Thereafter the induced band was excised from the SDS-PAGE gel, and injected into rabbits for antibody production. Third bleed of the serum showed positive signal with recombinant PfalMre11 C-terminus expression in bacteria, confirming the specificity of the antibody (Figure 17 C). The antibody was tested on parasite lysate using pre immune sera as a negative control. Anti- PfalMre11 antibody could detect a band corresponding to 145kDa, which is in good corroboration with PfalMre11 molecular mass (146 kDa) in *P. falciparum* 3D7. Such band was not seen in pre-immune sera (Figure 17 D).

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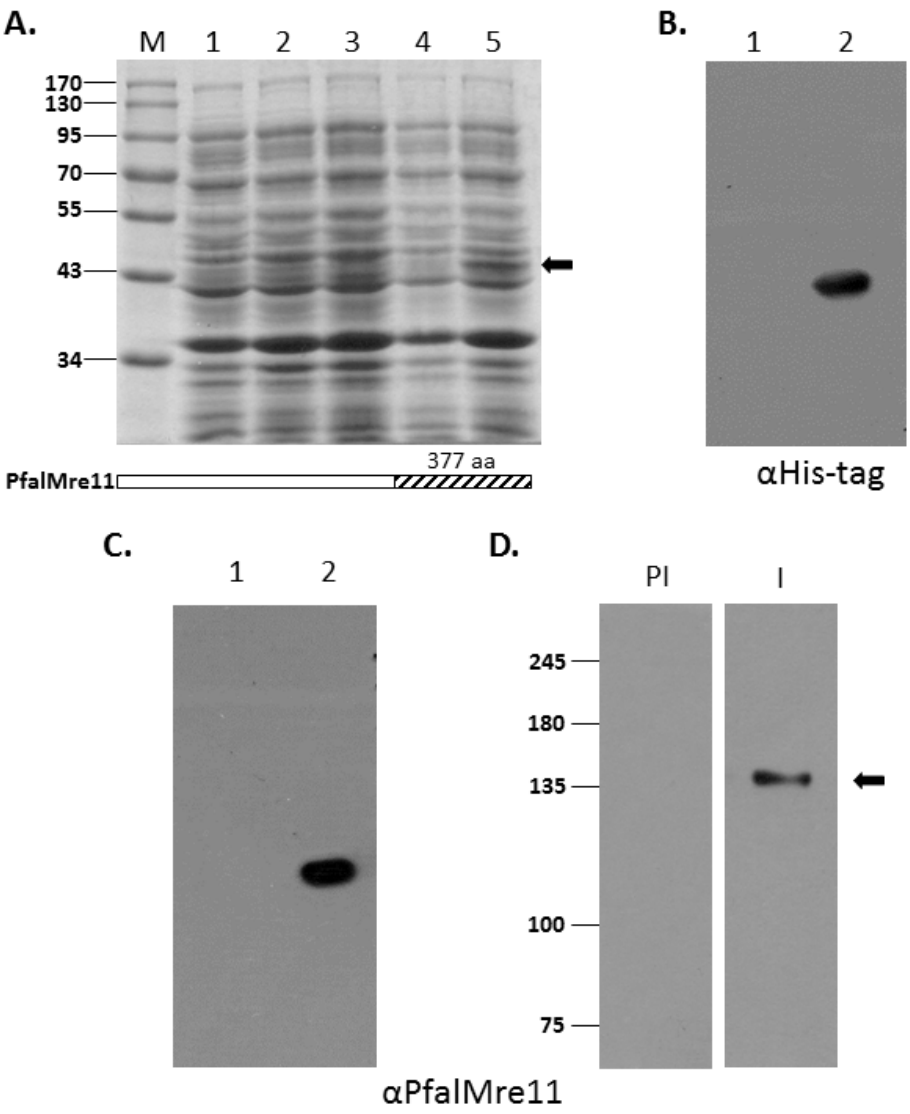

Figure 17: Expression of recombinant PfalMre11-C and antibody generation. A. polypeptide of 377aa corresponding to C-terminus domain (shown with hatched box) was expressed along with N-terminus His tag. Expression profile was monitored by collecting the cell lysate at time points 0hrs (lane 1) and 4hrs un-induced (lane 4) IPTG induction for 4hrs (lane 5). Lane 2 and 3 are loaded with cell lysate collected from sample carrying empty vector un-induced and IPTG induced sample for 4hrs. Protein ladder is presented on left. **B.** The recombinant His-PfalMre11 C expression is confirmed with anti-His antibody. Lane 1 is loaded with un-induced cell lysate and lane 2 is with IPTG induced cell lysate. **C.** The anti-PfalMre11 C antibody detected PfalMre11 C expression in bacterial cell lysate. Lane 1 represents un-induced sample and lane 2 represents induced sample. **D.** The anti-PfalMre11 antibody detects a parasite protein corresponding 145kDa (shown by an arrow) PI: Preimmune sera, I: Immune sera.

3.6 PfalMre11 expression is regulated in developmental stages of erythrocyte life cycle:

We have used Real-time PCR and western blot tools to estimate the expression levels of PfalMre11 during developmental stages of erythrocyte life cycle. We have synchronized the parasites, RNA isolation and parasite lysates were prepared at ring, trophozoite and schizont stages. Initial observation using semi-quantitative RT-PCR data showed that the steady-state levels of the *PfalMRE11* transcripts abundantly present in the trophozoite and schizont stages compared to ring stage (Figure 18 A). We have used endogenous gene *PfARP* as loading control and its expression was not altered throughout the erythrocyte developmental stages. We found that *PfalMRE11* transcript expressed 7 fold more in trophozoite stage and 5 fold more abundant in schizont compared to ring stage (Figure 18 B).

PfalMre11 protein expression levels were, detected by using anti- PfalMre11 antibody, found to be consistent with RT-PCR data (Figure 18 C). PfHsp70 protein was used as loading control as its expression remained unchanged during erythrocyte developmental stages of the parasite. Quantification of the protein levels were done by using Image J software. The steady state levels of PfalMre11 were 3 times more in trophozoite and 2.5 times more in schizont stage compared to ring stage (Figure 18 D). Data suggest that the differential expression of the PfalMre11 at transcript and protein level is developmentally regulated and the expression levels are controlled at transcript level.

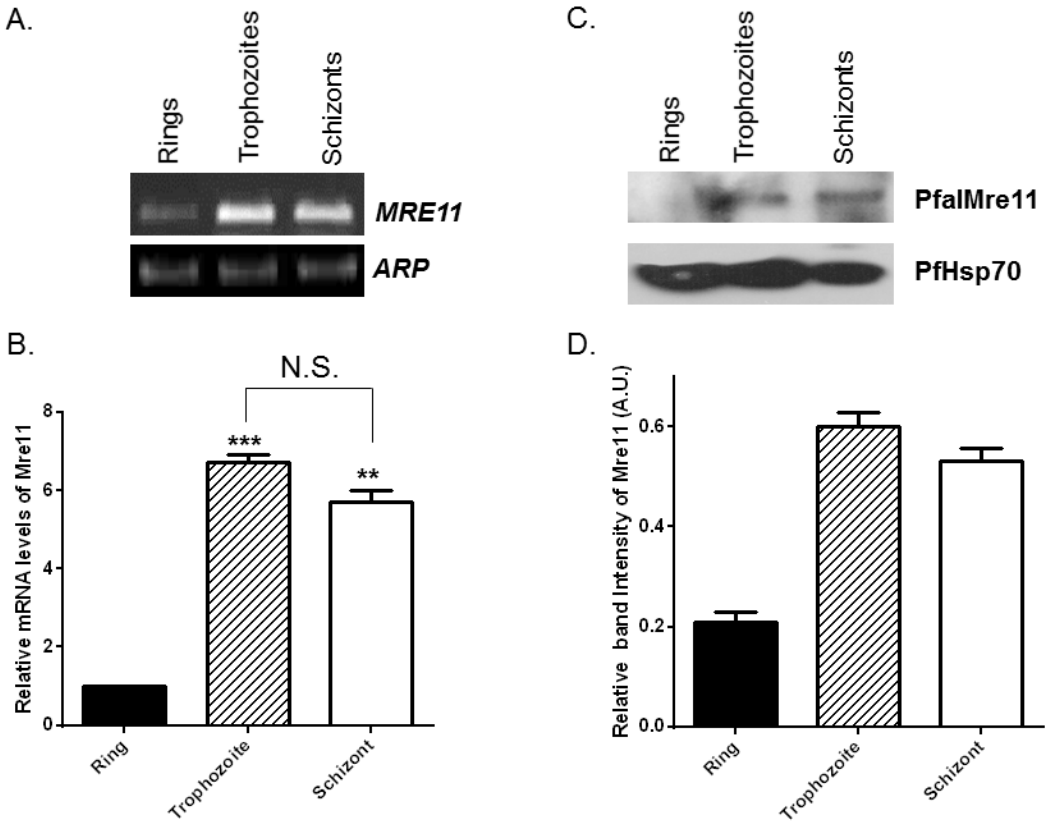

Figure 18: Stage specific expression profile of PfalMre11 during Intra-erythrocyte life cycle. A. Semi-quantitative RT- PCR showing *PfalMRE11* transcript levels at ring, trophozoite and schizont stages. *PfARP* served as loading control. **B.** Quantification of *PfalMRE11* transcript levels by real-time RT-PCR. Data was normalized with *PfARP*. The mean values ± SD provided from three individual expreriments. **C.** Expression profile of PfalMre11 protein against anti-PfalMre11 antibody in blood stages. PfHsp70 used as loading control. **D.** Quantification of western blots. Data was normalized against PfHsp70. *P* value was calculated using two tailed student's t- test. (** means $P < 0.01$; *** means $P < 0.001$; N.S means not significant)

3.7 DNA damage induced expression of PfalMre11 during intra- erythrocytic development:

Previous studies in yeast and mammals have shown that Mre11 respond to the DNA DSBs and acts as sensor protein and activates DDR pathway (Mimitou et al. 2009). It was shown that Mre11 gets up regulated upon DNA damage (Rolfsmeier et al. 2010 & Gasch et al., 2001). We sought to check the expression levels of *PfalMRE11* upon DNA damage. In that context, we have synchronized the parasite culture and made RNA and protein prep at ring, trophozoite and schizont stages for untreated and treated cultures (incase of treated culture, samples were isolated after 6hrs MMS induced DNA damage), to analyze *PfalMRE11* expression levels. Semi-quantitative analysis showed that *PfalMRE11* is up-regulated with endogenous control *PfARP* (Figure 19 A). Real-time PCR data revealed that *PfalMRE11* was up regulated to the given DNA damage, quantitatively *PfalMRE11* expression was 6 folds more at ring stage and 2 fold more at trophozoite, schizont stages than the expression levels of PfalMre11 in control samples (Figure 19 B). *PfARP* served as loading control as it remained similar at expression levels after DNA damage.

Next, we examined the protein levels of PfalMre11 after DNA damage at ring, trophozoite and schizont stages. Expression of PfalMre11 was significantly increased in all the stages with respect to untreated culture (Figure 19 C). Quantification of PfalMre11 showed nearly 2 fold increase in expression at all the developmental stages. PfHsp70 served as loading control (Figure 19 D). It also suggests that PfalMre11 induced expression may not be regulated at the protein level.

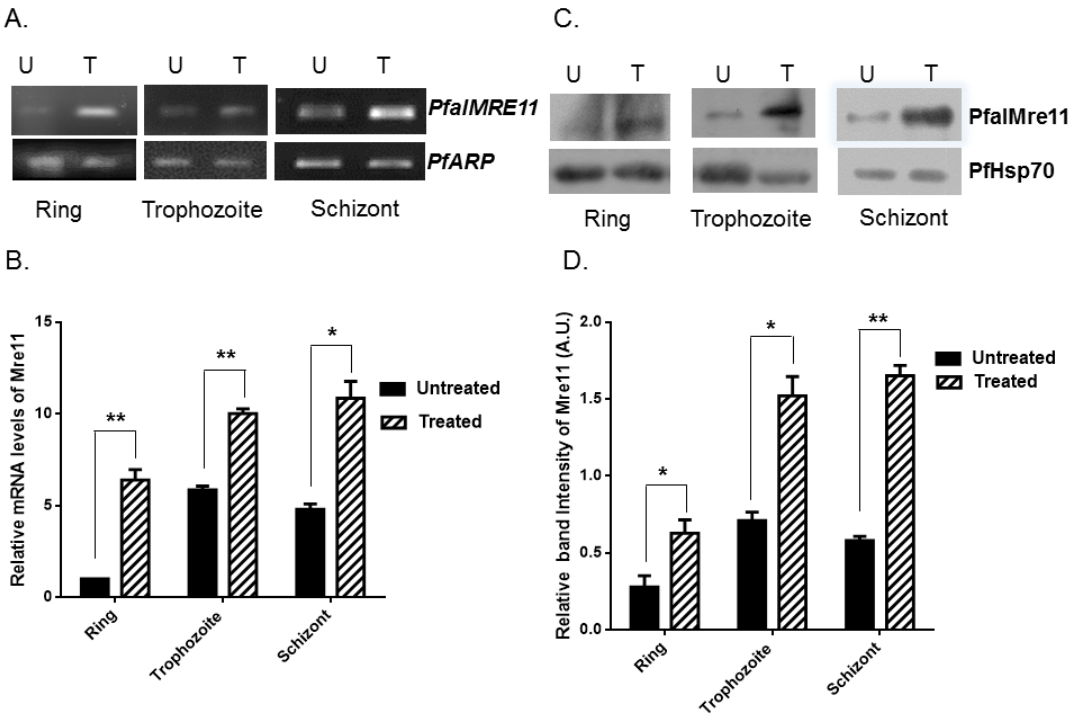

Figure 19: DNA damage induced upregulation of PfalMre11 during intra-erythrocyte life cycle. A. *PfalMRE11* transcript levels were compared at all the growth stages, *PfARP* served as loading control. *Plasmodium* cultures were treated with 0.05% MMS for 6hrs at ring, trophozoite and schizont stages. Semi-quantitative RT-PCR showed up-regulation of PfalMre11 transcript when compared between untreated (U) and MMS treated mRNA levels with respect to PfARP, loading control. **B.** *PfalMRE11* up-regulation was quantitatively measured by real-time RT-PCR. Data was normalized with respect to *PfARP*. Each sample mean value ±SD represented from three individual experiments. **C.** Western blot analysis showing PfalMre11 protein levels at ring, trophozoite and schizont stages for untreated (U), and 0.05% MMS treated (T) parasite lysate samples. PfHsp70 served as loading control. **D.** The quantification of western blots protein level in fold change by Image J. Protein expression folds were compared between untreated (U) and 0.05% MMS treated (T). Data was normalized with PfHsp70. The mean density ±SD values were taken from three individual expreiments. The *P* value was calculated by using two tailed student's t- test (* means $P < 0.05$; and ** means $P < 0.01$)

3.8 PfalMre11 failed to complement *Δmre11* deletion mutant in heterologous system *S. cerevisiae*:

Our initial observation in *Plasmodium* gave evidence that PfalMre11 ORF might be having a functional role in DNA damage repair. Therefore, we sought to investigate whether PfalMre11 is a bonafide ortholog of Mre11. We have used *S. cerevisiae* as a surrogate system to study the functional activities of PfalMre11 as genetic studies are difficult to carry out in *Plasmodium*. We have performed MMS sensitivity assay to determine whether PfalMre11 could complement yeast ⊿*mre11* mutant. For that, we have generated strains BSB3, BSB2 and BSB8 by transforming pTA: *PfalMRE11*, pTA: *ScMRE11* and empty plasmids into the Δ*mre11* strain respectively. It is known that Δ*mre11* is hypersensitive to MMS, the rescue of cell survival phenotype of BSB2 indicate complementation of the Δ*mre11*. We found that PfalMre11 could not complement Δ*mre11* (Figure 20).

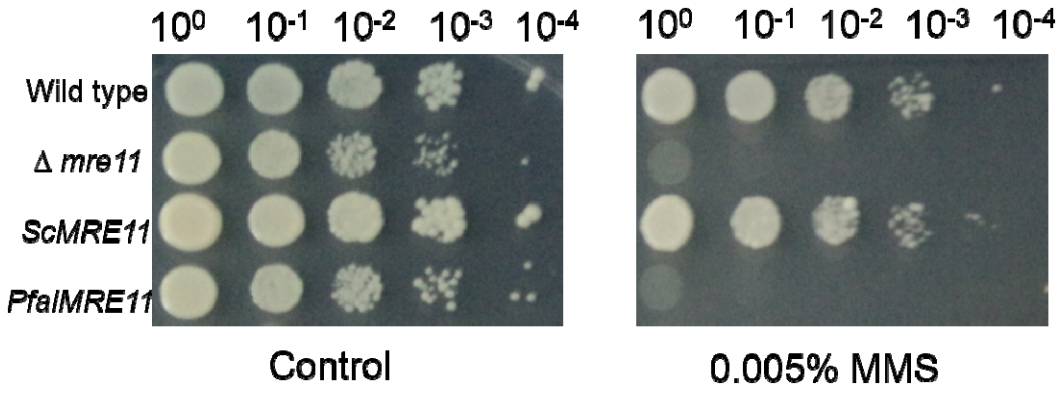

Figure 20: MMS sensitivity assay. All the four strains wild type (BSB1), *Δmre11* (BSB8), *ScMRE11* (BSB2), *PfalMRE11* (BSB3) were grown to OD_{600} 0.5 and spotted on synthetic medium plates lacking tryptophan by serial dilution. The spotting was done on plates containing 0.005% MMS and on no MMS plates.

3.9 PfalMre11 nuclease domain in conjugation with DDR domain of ScMre11 complements *Δmre11* deletion mutant in *S. cerevisiae*:

ScMre11 nuclease and DDR functions are attributed to two distinct domains: nuclease functions are associated with N-terminus and DDR functions are allotted to C-terminus. Both the domains are required for DNA DSBs repair and trans complementation studies showed that Mre11 functions can be separable (Bhatacharyya et al. 2008). Our previous observations suggest that PfalMre11 nuclease domain has considerable homology with ScMre11 nuclease domain (32%) and shows structural similarity in homology model and the C-terminus domain is highly divergent with Mre11 ortholog. We proposed that presence of evolutionarily conserved residues critical for nuclease function at phosphodiesterase motifs in PfalMre11 likely possesses nuclease function and the divergent C-terminus may not be able to interact with DDR protein machinery in a heterologous system. To prove these speculations we have constructed several chimeras by the fusion of PfalMre11 domains and ScMre11 domains (Figure 21 A). These constructs were transformed into *⊿mre11* yeast strain (MKB7) to check functional complementation. PfalMre11 chimera 1 is a combination of PfalMre11 N-terminus (1-832 amino acids) and C-terminus of ScMre11 (315-692 amino acids), chimera 2 construct is with N-terminus of ScMre11 (1-256 amino acids) and C-terminus PfalMre11 (727-1132 amino acids) and the other complementation construct was having C-terminus ScMre11. All the chimera constructs were generated after analysis of secondary structures of the individual proteins and based on nuclease domain sequence homology (secondary structures were analyzed by PSIPRED tool). All the recombinant construct expression was confirmed by checking transcript levels as the protein levels were not able to be detected with anti-*Myc* antibody (Figure 21 B).

To test the functional activities of these constructs individually in *⊿mre11* deletion strain (designated as BSB2 –BSB8), we performed complementation assays. We have checked MMS sensitivity for all the strains by two different assays. One is spotting assay, where different

dilutions of the yeast strains were spotted on MMS containing plates and grown for 72 hrs to check the cell survivability and in the second assay all the cultures were briefly exposed to MMS for two hours in mid-log phase (OD_{600} 0.5-0.7) and then brought back to grow on respective drop out plate without MMS by spreading equal number of cells. Cell survivability was scored after 72 hrs. The first experiment explains the ability of the cells to rescue the DNA damage when continuously exposed to DNA damage. The second assay tells whether the cells could rescue the DNA damage even at brief DNA insults, which will tell the efficacy of the heterologous protein in DNA repair. From both the assays we found that chimera 1 could rescue the DNA insults by MMS in *⊿mre11* strain (BSB4), which is similar to *ScMRE11* strain (BSB2). We did not find such DNA damage rescue phenotype in chimera 2 (BSB5), and this strain behaved similar to that of *⊿mre11* strain (BSB8) in terms of MMS sensitivity. We have used another construct having only C-terminus of the ScMre11 (BSB6) to rule out the possibility that the DNA damage rescue phenotype is not solely depending on C-terminus ScMre11 and we found no cellular DNA damage rescue phenotype which served as negative control for our experiments (Figure 22 A). These findings suggest that PfalMre11 N-terminus possesses nuclease function and the DDR function of the C-terminus domain may not be conserved in heterologous system.

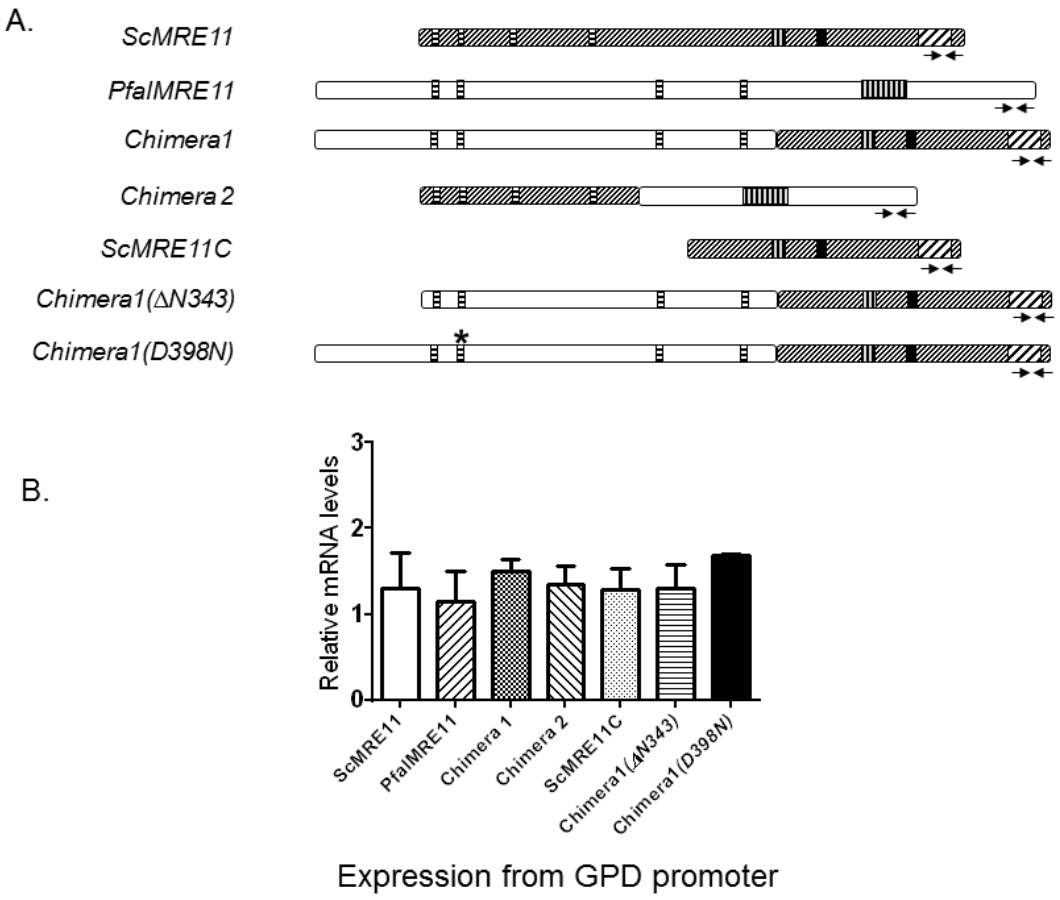

Figure 21: Construction of chimeras and their expression levels. A. Schematic representation of chimera fusion domains used for complementation studies. *ScMRE11*: full length sequence from *S. cerevisiae*, *PfalMRE11*: full length from *P. falciparum*, chimera 1: fusion of N-terminal sequence of PfalMre11 corresponding 1-832aa and C-terminal domain of ScMre11 corresponding 315-692aa. Chimera 2: fusion of N-terminal sequence of ScMre11 corresponding 1-256aa and C-terminal sequence corresponding 828-1233aa. *ScMRE11C*: gene fragment corresponding C-terminal domain 315-692aa. Δ343 chimera1: fusion of PfalMRE11 gene fragment corresponding to 344-828aa and the c-terminus gene fragment of ScMRE11 corresponding to 315-692aa. D398N chimera1: Chimera 1 gene fragment having missense mutation (D to N) corresponding to 398th amino acid in PfalMre11. **B.** Expression levels of all the constructs at mRNA level. Transcript were analyzed by real-time RT-PCR. Mean values (±SD) were taken from three individual experiments.

We sought to investigate the PfalMre11 N- terminus structure- function relationship. For that we generated two mutants in chimera 1; the first one was a missense mutation (D398N) and the other one was a deletion mutant (Δ1-343aa). Nuclease functions were characterized in ScMre11 by point mutation in phosphodiesterase motifs. Previously it was reported that replacement of aspartate (D) amino acid with asparagine (N) amino acid at the 56th position in the second phosphodiesterase motif nullifies the nuclease activity (Moreau et al. 1999). We have created mutation by corresponding amino acid substitution (D398N) using site directed mutagenesis. We had tested this mutant chimera 1 D398N by spotting assay and return to growth assay. We found that the mutant could not rescue the cell survivability, similar to *⊿mre11* strain (BSB8). Such drastic phenotype is unique for chimera1D398N. In yeast the corresponding mutant D56N shows mild sensitivity towards MMS (Moreau et al. 1999). Earlier we have described that the nuclease domain ranges from 350-796 amino acids and the N-terminus extension preceding nuclease domain of unknown function is absent in other Mre11 orthologs. Since it is associated with an unknown function, we deleted the unusual N -terminus extension to check whether this is dispensable for PfalMre11 N-terminus nuclease function. We generated a strain having *⊿343 chimera 1*(BSB7) and tested for DNA damage rescue phenotype. The deletion mutant strain BSB7 could not complement *⊿mre11* and is highly sensitive to MMS similar to BSB2. The data indicates that PfalMre11 N-terminus might be indispensable for nuclease functions attributed to PfalMre11 even though no motif like structure was found at the N-terminus extension sequence of PfalMre11.

The expressions of these mutants were confirmed at transcript level by doing Real-time PCR. The expression levels were found to be similar to that of chimera1 indicating the expression levels of the mutants did not alter. Return to growth assays for this mutant showed similar observations. Chimera 1 could complement *⊿mre11*, whereas chimera 2 and PfalMre11 highly sensitive to MMS, a phenotype similar to *⊿mre11*. Further, data indicates that PfalMre11 N – terminus nuclease domain in conjugation with ScMre11 C-terminus complements ScMre11

deficiency. Such phenotype was abrogated with the two mutations chimera 1 D398N and Δ343 chimera 1(Figure 22 B).

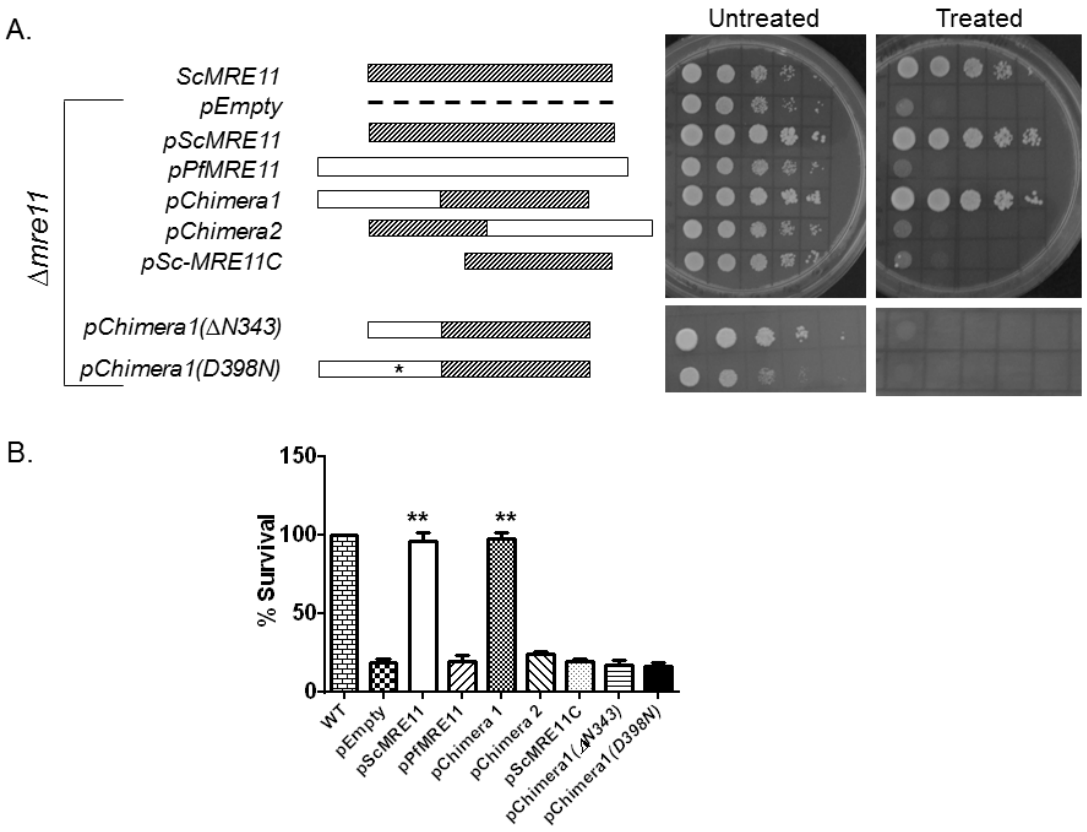

Figure 22: Functional complementation of ScMre11 by PfalMre11 nuclease domain in-conjugation with DDR domain of ScMre11. A. Spotting assay for the strains, BSB1-BSB7 and PVY1 on MMS containing and no MMS plates. The strains with respect to their constructs were presented on the left panel and the strain phenotype was observed on MMS and no MMS plates. **B.** Return to growth assay for the strains BSB1-BSB7 and PVY1. X- axis represent strains used in the assay, Y-axis represent % survivability of the cells after DNA damage. Mean value ± SD was provided after normalizing with untreated controls from four different experiments. The *P* value was calculated by using student's t-test. (** indicates $P< 0.01$)

3.10 PfalMre11 could form MRX complex in heterologous system:

It has been shown that in yeast and mammals Mre11 detects the DNA DSBs and recruit to the site in a complex. It interacts with two other proteins Rad50 and Xrs2 (yeast) or Nbs1 (human) in hetero-pentameric form, with two molecules of Mre11, two molecules of Rad50 and one of the Xrs2 /Nbs1. Complementation studies in the heterologous system showed that chimera 1 (or PfalMre11 N-terminus) could rescue the DNA DSBs which is indirect evidence that chimera 1 might form complex with heterologous complex proteins ScRad50 and ScXrs2. We were interested to investigate whether PfalMre11 is able to interact with heterologous MRX complex proteins to rule out the possibility that the lack of complementation is not due to the absence of the complex formation. We have used yeast two-hybrid system to investigate individual protein interactions. The system detects the interaction with the help of GAL4 transcription system where strong as well as weak interactions of the proteins can be assessed by cell survival phenotype. The strong interaction was detected using *ADE* reporter gene by cell survival phenotype on triple drop out plate (Sc-Ura-Leu-Ade), similarly weak interaction were detected with *HIS* reporter marker on triple drop out plate Sc-Ura-leu-His. We found that PfalMre11 could show homo-dimer formation and the interaction was robust (Figure 23 A). We tested ScRad50 interaction with chimera 1 and PfalMre11, and we found that ScRad50 showed interaction with both the Mre11 forms. However, the chimera 1 interaction was robust compared to PfalMre11 (Figure 23 C). We also tested for ScXrs2 with PfalMre11 and chimera 1 and we observed that both of them interacted with ScXrs2, although the interaction was feeble (Figure 23 D). Thus our results demonstrate that PfalMre11 may act as a homo-dimer which is consistent with other eukaryote Mre11 orthologs. The specificilty of interaction was confirmed by ruling out the possible interaction between ScMre11 and PfalMre11 (Figure 23 B). Interaction with ScRad50 and Xrs2 suggest that chimera 1 and PfalMre11 are able to form complex in heterologous system and act on DNA DSBs. PfalMre11 complex formation individually with

Xrs2 and ScRad50 indicates that it might exist as complex in *P. falciparum* even though the Xrs2 ortholog is not identified yet.

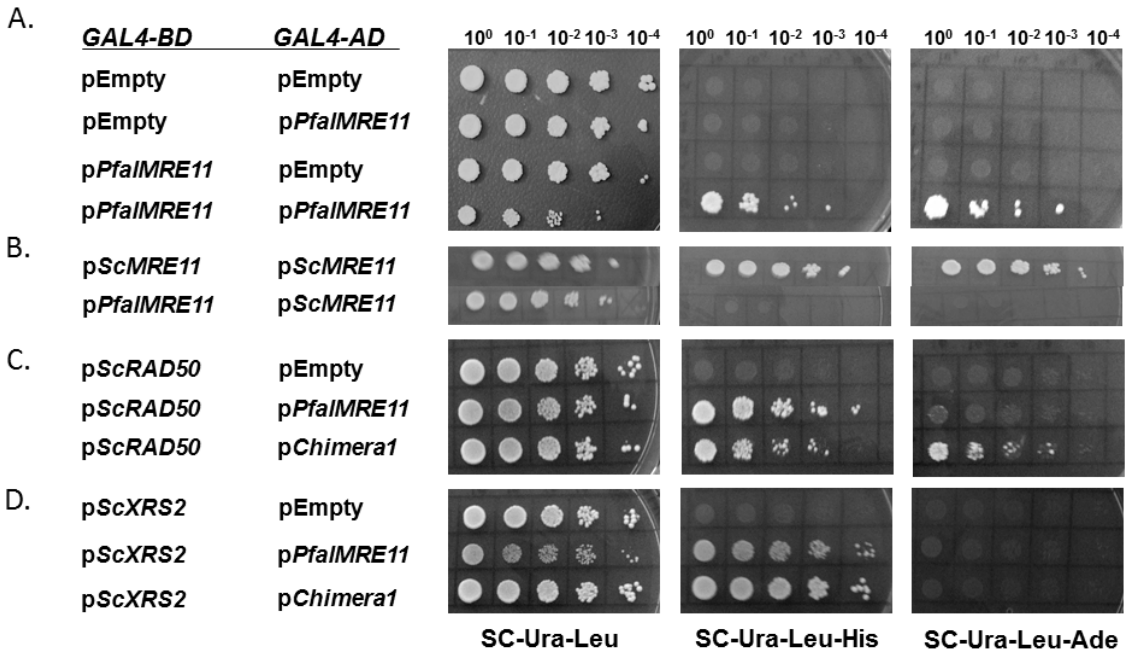

Figure 23: PfalMre11 could interact with yeast MRX complex proteins ScRad50 & ScXrs2. A. Full length PfalMRE11 ORF was fused to GAL4 AD domains in pGADC1, similarly PfalMRE11 ORF fused with GAL4 BD domain of pGBDUC1. Two-hybrid interactions were tested using yeast strain Pj694A, where ADE2 and HIS3 genes serves as reporter genes. All the strains were grown to same OD_{600} (1 OD_{600}) and serial dilutions of each strain was spotted on Sc-Ura-Leu plates lacking uracil and leucine to serve as control, and similarly samples were spotted on Sc-Ura-Leu-Ade and Sc-Ura-Leu-His plates laking either adenine or histidine, to test the protein interaction. **B.** *ScMRE11* or *PfalMRE11* ORFs were cloned into either pGADC1 or pGBDUC1 and the interactions were observed on respective synthetic complete medium plates. **C.** ScRad50 was fused with GAL4-BD domain in pGBDUC1 vector and the interaction were scored. **D.** ScXRS2 was fused with GAL4-BD domain and the interaction with *PfalMRE11* and chimera 1 was fused with GAL4-AD domain was monitored on synthetic complete medium plates lacking uracil, leucine and adenine or histidine.

3.11 PfalMre11 N-terminus (Chimera 1) activates NHEJ activity in heterologous system:

Previously it has been demonstrated that Mre11 acts as upstream sensor protein to DNA DSBs repair pathways and it is also crucial for both the HR and NHEJ pathways. In yeast, DSBs are majorly repaired by HR and in humans it is through NHEJ. Since our results demonstrate the role of PfalMre11 in DNA DSBs repair we sought to test the NHEJ activity in a heterologous system. We performed plasmid end joining assay, where plasmid recirculation would be the read out for NHEJ activity (Figure 24 A). We transformed equal amount of the linearized plasmid into the strains BSB2-BSB4 and PVY1 and uncut plasmid served as control. Total number of the transformants was calculated as the ratio of cut plasmid to the uncut plasmid. We found that chimera 1 showed moderate NHEJ activity compared to ScMre11 whereas ScMre11 NHEJ activity was similar to that of control. When compared with PfalMre11, NHEJ activity was not significant as seen for chimera 1. We also tested the NHEJ activity for chimera 1 D398N, which did not abrogate the NHEJ activity. These observations demonstrate that nuclease activity is not required for NHEJ activity which is consistent with previous findings (Moreau et al. 1999). NHEJ activity is activated by interaction of Mre11 with Ku protein (Daley et al. 2005), therefore, we investigated the interaction between Ku80 and Chimera 1 and with PfalMre11. We found that both PfalMre11 and chimera 1 interacted with yKu80, although the interaction was feeble (Figure 24 B). Therefore PfalMre11 might participate in NHEJ activity even though *Plasmodium* apparently lacks the classical NHEJ machinery. Recent findings suggest the presence of Alt-NHEJ in *P. falciparum* and the NHEJ activity found in heterologous system indirectly indicate that PfalMre11 might be having a role in Alt-NHEJ of *Plasmodium*.

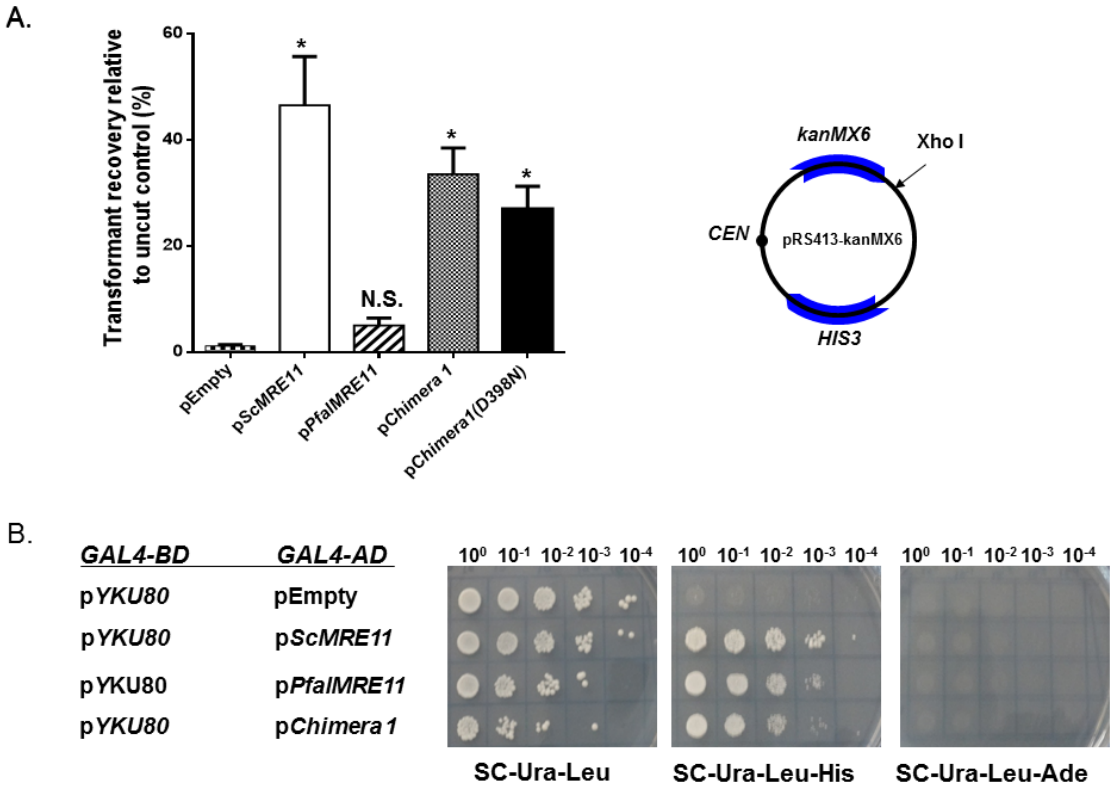

Figure 24: The N-terminal of PfalMre11 could take part in NHEJ activity in heterologous system. A. Bar graphical representation of NHEJ activity. X-axis represents different strains used for NHEJ assay, Y-axis represents % of the cell survivability, the read out is directly proportional to plasmid circularization. Mean values (±SD) were taken from three independent experiments. The *P*- values were calculated using student's t-test. (* indicates $P< 0.05$ and N.S means not significant). B. YKu80 interacts with PfalMre11. YKu80 ORF was fused with GAL4-BD in pGBDUC1 vector and interaction was tested with ScMre11, PfalMre11 and, chimera 1 fused with GAL4-AD domain in pGADC1. ScMre11 used as positive control.

3.12 Primary sequence analysis of Rad50 homologue in *P. falciparum*:

The blast search for the Rad50 sequence with other eukaryotic model organisms revealed the presence of important domains in PfalRad50. We could find nearly conserved Walker A and Walker B motifs which are associated with the Mre11 interaction, CXXC motif, and signature motifs were identified (Figure 25). The ORF was PCR amplified using primers and cloned into a pTZ intermediate vector. The sequence revealed that the encoded PfalRad50 protein contains 2236aa at 267 kDa molecular size and having isoelectric point 8.83.

Homology score search with other organisms showed very low homology when compared with protozoa, yeast and humans, ranging from 17% - 24%. The primary structure analysis suggests the lack of high homology with the eukaryote model organism, which also indicates that *Plasmodium* is evolved with variable DNA repair elements. (Table-7)

3.13 PfalRad50 is up-regulated upon induced DNA damage in intra - erythrocytic phase of life cycle:

We have analyzed the presence of PfalRad50 transcript in developmental forms of the erythrocyte life cycle. We found that PfalRad50 abundantly expressed at trophozoite and schizont stages which are the most vulnerable stages for DNA damage (Figure 26 A). Such differential expression of PfalRad50 is likely to have role in parasite biology. We have induced DNA damage with MMS treatment and analyzed the transcript levels for PfalRad50. We found that PfalRad50 gets up-regulated to induced DNA damage which displays similar pattern observed for PfalMre11, suggesting that PfalRad50 might be having evolutionarily conserved functional role in DNA repair along with PfalMre11 (Figure 26 B).

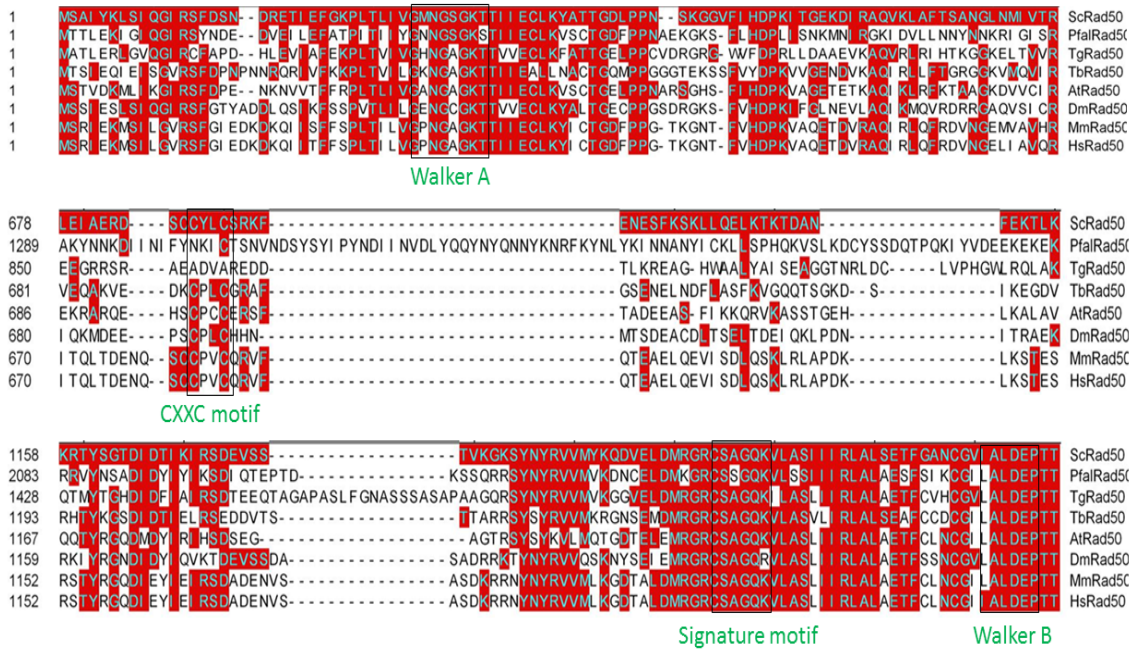

Figure 25: Primary structure analysis of PfalRad50. Multiple sequence alignment of PfalRad50 with budding yeast, *Toxoplasma gongii*, *T. bruci*, A. thaliana, *D.melanogaster* (fly), *M. musculus* (mouse) and human using CLUSTAL W method (Meg align, DNA star). Conserved motifs are highlighted in boxes.

Table 7: Homology score of PfalRaD50 with other Rad50 orthologs

	ScRad50	PfalRad50	TgRad50	TbRad50	AtRad50	DmRad50	MmRad50	HsRad50
ScRad50	100	24.47	23.7	25.84	26.91	25.08	26.07	26.14
PfalRad50		100	17.01	19.87	22.26	21.7	23.55	24.01
TgRad50			100	24.24	25.3	22.76	25.46	23.09
TbRad50				100	24.32	23.07	25.53	25.46
AtRad50					100	24.32	28.96	28.89
DmRad50						100	27.52	27.44
MmRad50							100	92.23
HsRad50								100

Sc- *S. cerevisiae*; Pfal- *P. falciparum*; Tg- *T. gondii*; Tb- *T. brucei*; At- *A. thaliana*; Dm- *D. melanogaster*; Mm- *M. musculus*; Hs- *H. sapiens.*

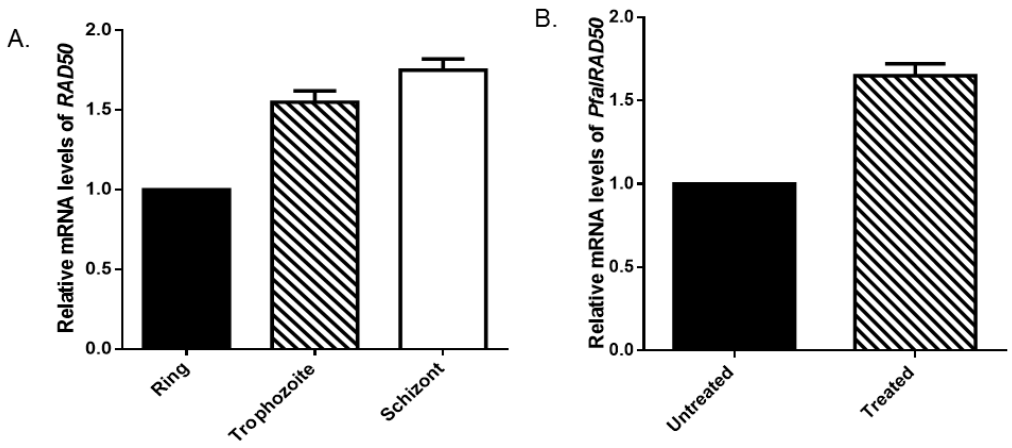

Figure 26: Stage specific and DNA damage induced expression of PfalRad50. A. PfalRad50 expressed in all the intra-erythrocyte developmental stages. Total RNA was isolated from ring, trophozoite and schizont stages. Real-time RT-PCR was used to quantify the expression levels. Expression levels were normalized with *PfRPA1* transcript. **B.** PfalRad50 expression was up regulated to induced DNA damage. Mixed stage culture majorly having trophozoite stage parasites were used for the assay. DNA damage was induced with 0.05% MMS for 6hrs, later total RNA was isolated from untreated and treated cultures. Real-time RT- PCR was used for quantification of transcript levels and the data was normalized with *PfARP1*.

3.14 PfalRad50 forms complex with Mre11:

Studies in model organisms shows that Mre11 forms a complex called MRX/N with Rad50 and Xrs2 (yeast)/ Nbs1 (human) and participates in DNA repair. *Plasmodium* genome sequence analysis and our studies on Mre11 in *Plasmodium* showed that Mre11 having evolutionarily conserved nuclease functions, therefore we thought to check the PfalMre11 and PfalRad50 interaction which will give an indirect evidence whether PfalRad50 having any role in DNA damage repair. We found that PfalRad50 shows weak interaction with PfalMre11 (Figure 27) which is similar to yeast Rad50 protein, suggesting that PfalRad50 acquired an evolutionarily conserved pattern of interaction and it participates in DNA repair.

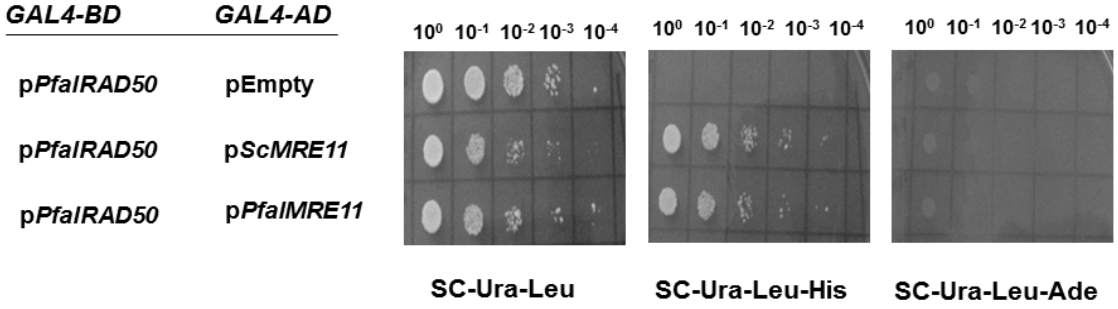

Figure 27: Interaction between PfalMre11 and PfalRad50. Yeast two-hybrid analysis of PfalRad50 with ScMre11, PfalMre11 and empty prey vector. Samples were spotted at different dilutions on triple drop out plates. His and Ade reporter genes were used for the assay.

3.15 Genome wide screening for identification of PfalMre11 interacting partners:

Plasmodium genome is composed of about 80% AT-rich content which hinders the expression and characterization of these genes in heterologous systems. Yeast two-hybrid system would offer the better expression of these genes, High-throughput yeast two-hybrid analysis with the cDNA library could not show any interacting domains for PfalMre11 (LaCount et al. 2005). Our observation on PfalRad50 interaction with PfalMre11 elicits the possibility for finding more PfalMre11 interacting partners. We have generated genomic library in prey vector using *Plasmodium* total genome (Figure 28). Library was transformed into Y2H yeast strain PJ69- 4A harboring bait vector with PfalMre11. The interacting partners were identified using selectable markers *ADH2* and *HIS3*. We could find about 32 strong interacting colonies. Plasmid rescue process could identify two of the interactors having the same fragment, we named it as Mre11 interacting protein 1 (MIP1) (Figure 29 A). BLAST analysis of the protein sequence does not show similarity with any of the DNA repair proteins and is unique for the parasite with hypothetical function (Data not shown).

3.16 *PfMIP1* expression profile in erythrocyte life cycle:

Initially, we have characterized *PfMIP1* interaction stringency by checking its auto-activation with bait plasmid. Further to characterize its expression in asexual life cycle, we have isolated total RNA from *P. falciparum 3D7* and analyzed the presence of *PfMIP1* at *Plasmodium* developmental stages. We found that *PfMIP1* expression is significantly more at schizont stage, which correlates with the expression profile of PfalMre11 (Figure 29 B). The data suggest that *Plasmodium* is evolved with different protein machinery to maintain genome stability.

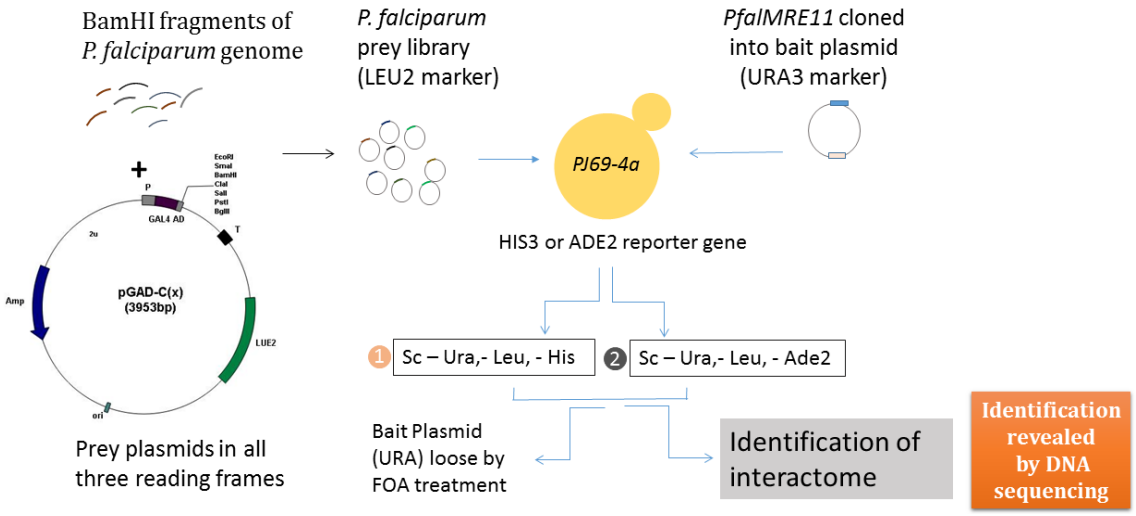

Figure 28: Schematic representation of construction of *P. falciparum* genome library in yeast-two hybrid prey vector.

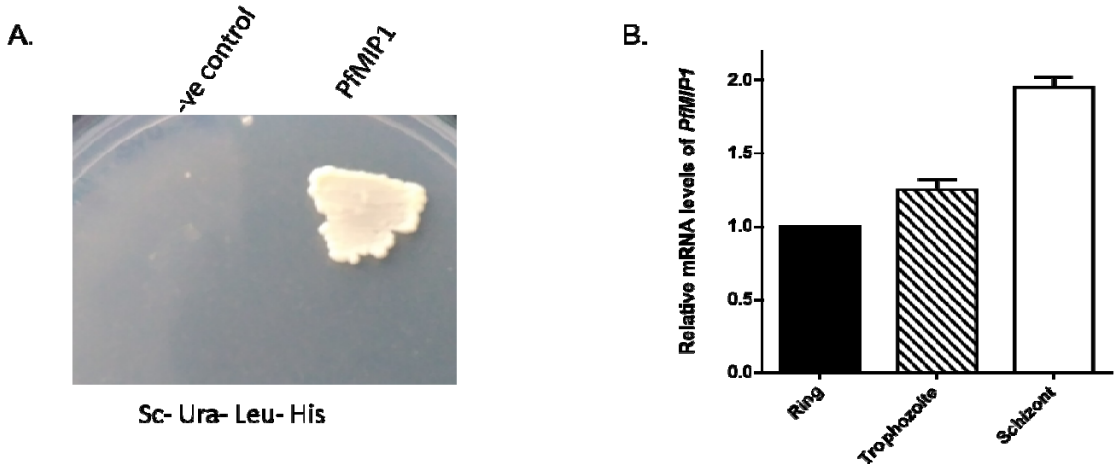

Figure 29: Expression of *PfMIP1* during intra-erythrocytic phase of parasite life cycle. A. Confirmation of PfMIP1 interaction with PfalMre11 by yeast two-hybrid analysis. PfMIP1 ORF identified in genome library screen was transformed with empty pGBDUC1 GAL4-BD vector to check the interaction, which served as negative control. **B.** *PfMIP1* transcript levels were quantified by real-time RT-PCR at ring, trophozoite and schizont stages. Transcript levels were normalized by *PfARP* transcript. Mean values (±SD) were taken from three individual experiments.

SUMMARY AND DISCUSSION

4.0 Discussion:

This work provides several insights into DNA repair pathways of *P. falciparum*. These are : i) existence of efficient DNA repair mechanisms in *Plasmodium*, ii) initial identification of functional role for Plasmodium DNA damage response genes, iii) characterization of central molecular player PfalMre11 nuclease activity in surrogate model yeast, iv) Characterization of PfalMre11 complex formation by yeast two-hybrid with heterologous as well as with *Plasmodium* protein PfalRad50, lastly v) identification of PfalMre11 interaction partners by screening *Plasmodium* genomic library.

Existence of DNA damage repair pathways are demonstrated in *P. falciparum* by UV DNA damage, which showed that different *P. falciparum* strains have differential DNA repair abilities (Excision repair and Mismatch repair) (Trotta et al. 2004). Here we showed that *P. falciparum* chloroquine sensitive strain 3D7 and resistant strain Dd2 do not show difference at global DNA DSB repair. We found that *P. falciparum* could repair UV DNA damage efficiently between 12 hrs to 24 hrs post damage, supporting the previous findings with MMS induced damage repair (Gopalakrishnan et al. 2013). In model eukaryotic organisms DNA damage response (DDR) pathway and DSB repair pathways are well established. However, genome annotation in *P. faliciparum* could not identify any genes involved in DDR except for *PfalMRE11* and *PfalRAD50* with very low homology score at protein level. Interaction of yeast Xrs2 with PfalMre11 as well as complex formation between PfalRad50 and PfalMre11 imply that there could be an equivalent of DDR pathway in *P. falciparum* as well, which is albeit poorly conserved when compared to other eukaryotes. Up-regulation of most of the putative DNA repair genes of *P. falciparum* in response to genomic insults indicates the existence of efficient DNA repair mechanisms in *Plasmodium*. Among these genes *PfalMRE11* is a crucial member of the DDR pathway and might act as sensor and modifier molecule. Our initial observation of DNA damage induced up-

regulation of *PfBLM* (RecQ1) and *PfWRN* are supported by their characterization by other research groups (Suntornthiticharoen et al. 2014; Rahman et al. 2015).

In eukaryotes Mre11p participates at different steps of DNA damage response, i) acts as sensor in DNA damage signaling, and ii) provides DNA substrates by initial resection of DSBs with intrinsic nuclease activity (Haber et al. 1999). We provide evidence that PfalMre11 actively participates in DNA damage repair during intra-erythrocyte growth stages upon induced DNA damage. In eukaryotes, DNA DSBs are majorly repaired by two DNA repair pathways, homologous recombination pathway, and non-homologous end joining pathway. Mre11p is found to be an upstream DNA repair protein which actively participates in HR and C-NHEJ/A-NHEJ (which is cell cycle phase specific). In *Plasmodium*, HR pathway was characterized and found to be the predominant pathway (Bhattacharyya et al. 2003 & 2005; Roy et al. 2014; Kirckman et al. 2013) for the repair of DSBs which is similar to *S. cerevisiae* DSBs repair pathway preference. However, the evidence for very low frequency repair by Alt-NHEJ depicts that the repair of DSBs at ring stage could be through Alt-NHEJ, although clear evidence is yet to be shown. The PfalMre11 expression at all the growth stages indirectly suggests that PfalMre11 might also play similar role in DSBs repair pathways. Recently it was shown that Mre11p nuclease activity helps in repair pathway choice during DSBs repair (Shibata et al. 2014). However in *Plasmodium*, it is still unknown.

Genetic manipulation in *Plasmodium* is technically difficult, so it poses a limit to carryout genetic studies in this system. Nevertheless, yeast serves as an excellent surrogate model to study the function of heterologous *Plasmodium* genes. We have taken the advantage of this model as the Mre11 ortholog shows considerable homology in the nuclease domain (33%). We have observed that PfalMre11 could not complement ScMre11 function in *⊿mre11* yeast strain. Chimera 1 which is the fusion of PfalMre11 N-terminus with ScMre11C-terminus DDR domain was able to rescue the DNA damage. Failure of ScMre11 C-terminus DDR domain alone in the rescue of

DSBs highlighted our finding that PfalMre11 N-terminus nuclease domain is evolutionarily conserved. The observation was confirmed by mutational analysis in the second phosphodiesterase motif at D398N, which corresponds to D56N residue in ScMre11 nuclease domain. PfalMre11 interaction with MRX complex proteins ScRad50, ScXrs2 in yeast two-hybrid provide the evidence that PfalMre11 participates in DNA damage response. NHEJ activity of PfalMre11 and the chimera1 in heterologous system suggest that PfalMre11is able to participate in NHEJ pathway and its nuclease function is not necessary for NHEJ activity which is also observed in the case of yeast ScMre11. The ability of PfalMre11 participation in NHEJ was further confirmed by the interaction between PfalMre11 and yKu80. PfalMre11 self-interaction and PfalRad50 interaction demonstrated that PfalMre11 form complex in *Plasmodium* as well during repair of DNA damage.

The A470 motif in ScMre11 is found to be conserved with other Mre11 orthologs except for dipterans and *Plasmodium*. A470 motif participates in telomere maintenance by loading telomerase to maintain the telomere length and elicits telomere end recombination to provide telomere end protection. In dipterans telomere, length maintenance is performed by transposable elements whereas in *Plasmodium* it is found to be by the telomerase mediated pathway. Thus, the absence of the motif A470 in *Plasmodium* puts forward the unidentified role of PfalMre11 in telomere length maintenance. Mre11 interacting proteins are well characterized in eukaryotes, by several methods. *Plasmodium* genome is covered with 60% hypothetical proteins and lack of the homology or poor homology with evolutionarily conserved proteins and unavailability of antibodies to majority of *Plasmodium* DNA repair proteins limits the direct testing for interacting partners. High throughput genome wide interaction studies using yeast two-hybrid system serves as an excellent tool. In *Plasmodium*, high throughput interaction studies were performed with cDNA library (LaCount et al. 2005) having the amplicon ranging from 300-400bp. It could not find any interacting protein network for PfalMre11 as well for PfRad50. While we could show

that PfalMre11 interacts with PfalRad50. In the high throughput analysis, such interaction was not identified, raising the possibility that cDNA library failed to cover all the interacting domains. Our genomic library approach enabled us to find interacting partners for PfalMre11. Although many of them are under screening process, we could identify one sequence having a hypothetical function named as *PfMIP1* specific for *Plasmodium*. The intra-erythrocytic expression profile of *PfMIP1* corroborates well with the expression profile of both *PfalMRe11* and *PfalRAD50*.

Mre11 is shown to be a crucial central molecule for DNA DSBs repair. Loss of Mre11 shows severe DNA damage sensitivity towards DNA damage agents, and deletion of Mre11 shows embryonic lethality in mammals and shows slow growth phenotype in yeast (Haber et al. 1999). In other protozoan *T. brucei* it was shown that TbMre11 is dispensable for growth and is essential for DNA repair activities (Tan et al. 2002). PfalMre11 nuclease activities are evolutionarily conserved with other Mre11 orthologs. We speculate that PfalMre11 might have an essential role in DNA repair pathways in *P. falciparum*. The essential role of PfalMre11 nuclease function in HR, and a likely involvement in Alt-NHEJ repair mechanism indirectly underscores its participation in all sorts of DSB repair pathways in *P. falciparum*. Nonetheless, generation of a PfalMre11 knockout mutant or an inhibitor would answer whether PfalMre11 is essential for the parasite growth and DNA repair activities.

PfalMre11 shares less homology with human Mre11 which allows the possibility to design inhibitor molecules for PfalMre11 and inhibit the DNA damage repair. So PfalMre11 can be used as a potential drug target to curb malaria.

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APPENDIX

Unicellular and multicellular organisms are continuously exposed to a multitude of agents that cause several types of DNA damage (Smeenk et al., 2013). DNA double strand breaks (DSBs) are the most severe kinds of DNA damage. Unrepaired or inaccurately repaired damages lead to genome instability and cause genetic disorders like ataxia telangiectasia, Nijmegen breakage syndrome, Bloom's syndrome and Werner's syndrome. Eukaryotic cells have evolved with different DNA repair mechanisms to maintain genome stability. DNA double strand breaks (DSB) are repaired by two major repair pathways: a) Homologous recombination (HR) (error free) and, b) Non homologous end joining (NHEJ) (error prone). DNA damage arrests the cell cycle progression at one of the cell cycle checkpoints: G1/S checkpoint before DNA replication; S-phase during replication and G2/M checkpoint before mitotic phase. Cell cycle arrest allows the cell to repair by one of the DNA repair pathways depending on the type of DNA damage (Lukas J et al., 2004). All these DNA damage repair activities are collectively known as DNA damage response. HR utilizes homologous template to repair DSBs during S and G2 phases of the cell cycle, while NHEJ repair pathway is active during all the phases of the cell cycle. Importantly, NHEJ is preferred in G1 phase where homologous chromatid is absent (Takata M et al., 1998; Delacote F et al., 2008). Rad52 epistasis group genes monitor HR (Symington et al., 2002), where Rad51 participates in the search of homologous sequence and strand invasion. NHEJ pathway involves direct ligation of two ends in association with Ku70/80, DNAPkcs, DNA ligase IV and XRCC4 (Dongliang et al., 2008). Single strand annealing (SSA) pathway also participates in the repair of DSBs which utilizes Rad52 and it is Rad51 independent pathway (Symington et al., 2002). DNA damage response that involves sensors, transducers and effectors, play regulatory roles during DNA repair. Sensor proteins (Mre11 and chromatin modifiers) detect the DNA damage and amplify the signal by activating transducer molecules (checkpoint kinases), effector protein cascade respond to the DNA damage and activates DNA damage repair mechanisms. Mre11 has multiple functions as it is involved in telomere length

maintenance (Bhattacharyya et al., 2008), promotes DNA repair activities with its nuclease function (D Amours et al., 2002) and DNA damage monitoring activities through ATM pathway (Paques et al., 1999). The Mre11 nuclease function is confined to the N-terminal domain and the DDR signaling function is attributed to the C-terminal domain through XRS2 interaction and these functions are separable in trans (Bhattacharyya et al., 2008). Recent reports show that Mre11 exonuclease and endonuclease activities can regulate the DSB repair pathway choice (Shibata A et al., 2014).

The apicomplexan protozoan parasite *Plasmodium falciparum* causes malignant malaria in humans. It undergoes sexual life cycle in *Anopheles* mosquito and asexual reproduction in the human host. Growing resistance to recent antimalarial drug artemisinin was observed (Dondrop et al., 2009) in *Plasmodium sps*. New prevention strategies need to be designed to control malaria which allows identifying new drug targets. *Plasmodium* resides within erythrocytes and utilizes hemoglobin as source of aminoacids. Parasites generate free radicals and reactive oxygen species during the process of hemoglobin degradation, a potential source for DNA damage (Henle ES et al., 1997). DSBs are the most lethal form of DNA damage, un-repaired DSBs would lead to the death of the unicellular organism (Frankenberg-Schwager and Frankenberg, 1990). *Plasmodium* parasite are continuously exposed to DNA damage during its asexual life cycle and *Plasmodium* being a unicellular organism targeting its double strand breaks repair mechanisms would be an excellent strategy. DSBs repair mechanisms are poorly understood in *Plasmodium* parasite. The existence of homologous recombination (Bhattacharyya et al., 2003) and an Alt-NHEJ pathway (Kirkman et al., 2013) were reported. Biochemical functions of PfRad51 an ortholog of Rad51 protein (Bhattacharyya et al., 2004), RPA protein (Voss et al., 2002) and PfRad54 protein (Gopalakrishnan et al., 2013) were characterized in *P. falciparum*. Studies in HR machinery with dominant negative of PfRad51 protein

showed that PfRad51 plays a crucial role in the repair of DSBs (Roy et al., 2014). In *Plasmodium* genome a putative PfalMre11 ORF was identified, its functional role was well studied in model organisms. As Mre11 plays multiple functions at different levels of DNA damage response and telomere length maintenance, targeting this upstream DNA repair molecule as drug target would be a good strategy.

In this work, three questions have been addressed. We asked whether *Plasmodium* parasites are sensitive to DNA damaging agents and can repair the DNA damage. Secondly, we have characterized one of the key molecular players of DNA damage response PfalMre11. Thirdly, we have constructed *Plasmodium* genomic DNA library to identify interacting partners of PfalMre11 through yeast two-hybrid screening.

We asked how parasite respond to DNA damaging agents? To answer this we have selected *P. falciparum* strains from different geographical regions: one is chloroquine sensitive African strain 3D7 and the second is chloroquine resistant Indo china strain Dd2, and induced DNA damage at different UV doses. We found that both *Plasmodium* strains have similar DNA damage sensitivity. We have compared the ability of DNA damage repair of *P. falciparum* strains with eukaryote unicellular model organism *S. cerevisiae* and found that *P. falciparum* strains are less sensitive to DNA damage which suggests that parasites are evolved with strong DNA repair mechanisms as it encounters DNA damage throughout their asexual life cycle (during detoxification of heme). We have studied the repair kinetics of DNA damage of *Plasmodium* nuclear and mitochondrial genomes and found that *Plasmodium* could repair DNA between 12hrs to 24hrs. DNA damage response genes are identified in *Plasmodium* genome through genome mining approach (Lee at al., 2014). We have selected a few of the DNA damage response molecules and verified their expression upon DNA damage induction. We found that most of the selected genes are up-regulated upon DNA damage,

suggesting they are likely to have roles in DNA repair. We have selected one such key DNA repair molecular player named PfalMre11 and characterized its functional roles.

We have compared the protein sequence of PfalMre11 with other eukaryotic Mre11 proteins where its functional roles were well studied. Homology sequence scores suggest that PfalMre11 exhibits poor identity with other Mre11 proteins. This observation raises the question that whether the putative PfalMre11 is a genuine ortholog of Mre11. We have expressed the C-terminal part of recombinant PfalMre11 and raised antibody. We used this antibody to check the expression profile of PfalMre11 during the asexual life cycle. We found that PfalMre11 RNA and protein are abundantly present at trophozoite and schizont stages which are prone to DNA damage. We have investigated the induced expression of PfalMre11 in response to DNA damage, where we found that PfalMre11 is up-regulated in all the growth stages, suggesting that although PfalMre11 participates in DNA damage repair. We sought to characterize functional activities of PfalMre11. In-silico analysis suggested that although PfalMre11 displays very less identity score with other eukaryote Mre11 proteins, the critical residues of the nuclease domain are conserved. In other systems, it was shown that mutations in these residues abrogate nuclease function of Mre11 protein (Krogh et al., 2005). The presence of unique N-terminal extension sequence and low complexity regions are found in PfalMre11 protein. Homology model generated for nuclease domain of PfalMre11 resembles nuclease domain of ScMre11. These observations allowed us to study the functional characteristics of PfalMre11 in heterologous yeast system W3O3. We have expressed PfalMre11 in *∠mre11* yeast strain and performed MMS sensitivity assay. We found that PfalMre11 failed to complement *∠mre11*. We speculated that one of the domains of PfalMre11 may not be functional in the heterologous system. Thus, we have generated chimeras by fusion of different domains of PfalMre11 and ScMre11. We fused N-terminal domain of PfalMre11 with the C-terminal domain of

ScMre11 to generate Chimera 1 and Chimera 2 is the fusion of N-terminal domain of ScMre11 with the C-terminal domain of PfalMre11. We have performed two assays a) MMS sensitivity assay b) Return to growth assay to check whether any of the chimeras would complement ⊿*mre11*. We found that Chimera 1 could complement the ⊿*mre11* functions suggesting that the N-terminal domain function is evolutionarily conserved. In eukaryote model organisms, Mre11 N-terminal domain participates in nuclease activity. To prove that the N-terminal domain of PfalMre11 possesses nuclease activity, we have generated two kinds of mutations: one is the deletion of an N-terminal unique sequence of 343 amino acids and the second is a point mutation in the 2nd phosphodiesterase motif of PfalMre11. Either of these constructs failed to complement ⊿*mre11* functions. This data suggests that PfalMre11 nuclease functions are evolutionarily conserved.

Next we asked whether PfalMre11 can form complex with ScRad50 and ScXrs2. We have used yeast two-hybrid assay to investigate protein- protein interaction. We found that PfalMre11 is able to form a dimer, and it could weakly interact with ScRad50, and ScXRS2. This data suggests that PfalMre11 participates as complex during DNA repair. We have tested the role of PfalMre11/ chimera 1 and Chimera 1 (D398N) in NHEJ activity in the heterologous system. We found that Chimera 1 could perform NHEJ activity. We were interested to see whether PfalMre11 exists in a complex with PfalRad50 in the parasite. Protein interaction study between PfalMre11 and PfalRad50 suggests that PfalMre11 is able to form complex. We have also checked the expression profile of PfalRad50 in erythrocyte growth stages. We found a good corroboration with the PfalMre11 expression profile. We have investigated DNA damage induced expression of PfalRad50 and found that PfalRad50 is upregulated upon DNA damage. These observations indicate that PfalRad50 is likely to participate in DNA repair along with PfalMre11. These observations allowed us to check the other interacting

partners of PfalMre11. To this end we have constructed *P. falciparum* gDNA yeast two-hybrid library. Yeast two-hybrid analysis revealed one of the *Plasmodium* protein with hypothetical function interacts with PfalMre11 and its temporal expression was comparable with the expression profile of PfalMre11 in developmental growth stages of erythrocyte asexual life cycle.

Thus, our studies reveal that *P. falciparum* strains geographically distributed in different regions possesses effective DNA repair pathways. One of the key molecular players of DNA repair, namely PfalMre11 was characterized. We have also identified a novel interacting partner of PfalMre11. Both PfalMre11 and its novel interacting partner exhibit poor sequence identity with human proteins, suggesting that they could be studied further as potential drug targets.

Publication:

Identification of *Plasmodium falciparum* DNA repair protein Mre11 with an evolutionarily conserved nuclease function.

Sugith Babu Badugu, Nabi SA, Vaidyam P, Laskar S, Bhattacharyya S, Bhattacharyya MK.
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PUBLICATIONS

PLOS ONE

RESEARCH ARTICLE

Identification of *Plasmodium falciparum* DNA Repair Protein Mre11 with an Evolutionarily Conserved Nuclease Function

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Abstract

The eukaryotic Meiotic Recombination protein 11 (Mre11) plays pivotal roles in the DNA damage response (DDR). Specifically, Mre11 senses and signals DNA double strand breaks (DSB) and facilitates their repair through effector proteins belonging to either homologous recombination (HR) or non-homologous end joining (NHEJ) repair mechanisms. In the human malaria parasite *Plasmodium falciparum*, HR and alternative-NHEJ have been identified; however, little is known about the upstream factors involved in the DDR of this organism. In this report, we identify a putative ortholog of Mre11 in *P. falciparum* (PfalMre11) that shares 22% sequence similarity to human Mre11. Homology modeling reveals striking structural resemblance of the predicted PfalMre11 nuclease domain to the nuclease domain of *Saccharomyces cerevisiae* Mre11 (ScMre11). Complementation analyses reveal functional conservation of PfalMre11 nuclease activity as demonstrated by the ability of the PfalMre11 nuclease domain, in conjunction with the C-terminal domain of ScMre11, to functionally complement an *mre11* deficient yeast strain. Functional complementation was virtually abrogated by an amino acid substitution in the PfalMre11 nuclease domain (D398N). PfalMre11 is abundant in the mitotically active trophozoite and schizont stages of *P. falciparum* and is up-regulated in response to DNA damage, suggesting a role in the DDR. PfalMre11 exhibits physical interaction with PfalRad50. In addition, yeast 2-hybrid studies show that PfalMre11 interacts with ScRad50 and ScXrs2, two important components of the well characterized Mre11-Rad50-Xrs2 complex which is involved in DDR signaling and repair in *S. cerevisiae*, further supporting a role for PfalMre11 in the DDR. Taken together, these findings provide evidence that PfalMre11 is an evolutionarily conserved component of the DDR in *Plasmodium*.

Introduction

Malaria continues to be one of the deadliest infectious diseases worldwide, resulting in nearly several millions deaths annually. *P. falciparum*, a mosquito-borne protozoan parasite, is responsible for most malaria deaths. Infection with *P. falciparum* can lead to serious medical complications, including cerebral malaria, as well as increased risk for long-term neurological and cognitive impairments. Currently, no malaria vaccine is available but effective treatments do exist. However, the rapid emergence of drug-resistant *P. falciparum* [1] underscores the urgent need for additional pharmacotherapies that are effective.

DNA repair pathways represent potential sources of new targets for treatment of *Plasmodium* infections, given that even a single un-repaired DSB leads to death of a unicellular organism [2]. In fact, previous research has shown that the parasite is susceptible to extensive DSBs caused by exposure to radiomimetic drugs, accumulation of free heme, innate host immune responses and DNA replication errors [3–5]. In eukaryotes, DSBs activate the DDR pathway which recognizes and processes DSBs, activates cell signaling pathways, and facilitates repair by either NHEJ or HR. In *P. falciparum*, HR has been identified and characterized [6, 7], is the predominant DSB repair mechanism [8], and is essential for repairing DSBs [9]. Interestingly, *P. falciparum* appears to lack the canonical NHEJ pathway consisting of the Ku heterodimer, DNA-PKc, DNA ligase VI and XRCC4, and alternative NHEJ (A-NHEJ) is utilized at a very low frequency [8]. The presence of HR and A-NHEJ in *Plasmodium* suggest potential overlap with DNA repair pathways in well-characterized eukaryotes; however, the factors involved in *Plasmodium* DDR remain largely unknown and orthologs of key eukaryotic DDR factors including ATM (yeast Mec1), ATR (yeast Tel1), Chk1, Chk2 (yeast Rad53) and Mre11 are yet to be identified and characterized.

The multi-functional Mre11-Rad50-Xrs2/Nbs1 (MRX/N) complex (*NBS1* is the vertebrate ortholog of yeast *XRS2*) plays pivotal roles in DNA repair [10] through NHEJ and mitotic HR. MRX is also required for the formation and processing of DSBs necessary for the proper disjunction of paired chromatids during meiotic homologous recombination [11, 12]. During mitotic DSB repair, the MRX complex has two distinct roles: (a) it acts in DNA damage signaling as an upstream DNA damage sensor and modifier in the ATM (Tel1 in yeast) pathway [12], where it activates Tel1p, which in turn specifically phosphorylates Mre11p and Xrs2p in response to DNA damage; and (b) it activates DNA repair, possibly through Mre11p nucleolytic processing [13]. Mre11p has multiple *in vitro* nuclease activities including 3'-5' exonuclease on dsDNA substrate and endonuclease activity on ssDNA. However, none of these activities explain the generation of MRX dependent 3' overhangs found *in vivo* during mitotic and meiotic DSB processing [14–16]. It has been speculated that the limited DNA unwinding activity of Mre11-Rad50-Nbs complex in conjunction with the multiple nuclease activities of Mre11p may be responsible for 3' overhang generation [14]. A recent study showed that the repair choice between NHEJ and HR is directed by distinct Mre11 nuclease activities. While, inhibition of endonuclease activity promoted NHEJ in lieu of HR, inhibition of exonuclease activities conferred a general repair defect [17]. Mre11p nuclease activities are confined to its N-terminal domain, while the C-terminal region contains an Xrs2p binding site and confers Tel1p-mediated DDR activity that is separable from the essential nuclease activity in trans [18]. In addition to its role in DSB repair, Mre11 plays important roles in several aspects of telomere maintenance [19–30]. For example, Mre11p nuclease activity and DNA damage signaling are both required for telomerase mediated telomere formation [18].

Among the protozoan parasites, Mre11 has been identified and characterized in *Trypanosoma brucei* and shown to influence HR and DSB repair [31]. However, it is dispensable for HR mediated VSG gene duplication [32]. In this study, we report molecular cloning of

Plasmodium falciparum Mre11 *(PfalMRE11)* and show up-regulation of PfalMre11 in response to DNA damage. Additionally, through yeast complementation experiments, we demonstrate that PfalMre11 possesses nuclease activity at its amino-terminal domain. Finally, we show that PfalMre11 interacts ScXrs2, suggesting involvement of PfalMre11 in *Plasmodium* DDR.

Materials and Methods

Parasite culture and Methyl Methanesulfonate (MMS) treatment

P. falciparum 3D7 culture was maintained in RPMI1640 media (5% hematocrit) supplemented with 1% Albumax (Invitrogen) and 0.005% hypoxanthine at 37°C using the candle jar method. Parasite cultures were divided into two equal portions: one portion was treated with 0.005% MMS for six hours and a non-MMS treated portion was grown in parallel for the same amount of time. Total RNA/ protein were isolated from both treated and untreated cultures for RT-PCR and Western analysis.

Plasmids

Sequences of all PCR primers used in this paper are presented in S1 Table. Using *P. falciparum* genomic DNA as a template, full length *PfalMRE11* was amplified using OMKB23 and OMKB24 as the forward primer and the reverse primer, respectively, both of which had BamHI flanking sequences. The PCR amplified product was cloned in 2μ yeast expression vector pTA [33]. Similarly, full length *ScMRE11* and the *ScMRE11* C-terminal domain (378 amino acids) were amplified using *S. cerevisiae* genomic DNA as a template and the primer-pairs OMKB84-OMKB85 and OMKB161-OMKB85, respectively. Each primer-pair contained a BamHI flanking sequence in the forward primer and PstI flanking sequence in the reverse primer. The amplified products were cloned into the pTA vector. The cloned vectors with full length and C-terminal domain of *ScMRE11* are referred as *ScMRE11* and *ScMRE11C*, respectively. Next, we constructed a chimera with the N-terminal domain of *PfalMRE11* (832 amino acids) fused to the C-terminal domain (378 amino acids) of *ScMre11*. This was done by amplifying a 2,496 bp stretch of DNA corresponding to the N-terminal region of PfalMre11 with primer-pair OMKB23-OMKB100 (HindIII site), and amplifying a 1,134 bp stretch of DNA corresponding to the C-terminal region of ScMre11 with the primer-pair OMKB98 (HindIII site)-OMKB85 (PstI site). The PCR amplified products were fused and cloned into pTA vector and the resulting plasmid is referred in this paper as as *Chimera 1*. To create an N-terminal deletion of 343 AA in *Chimera* 1, i.e. Chimera *1 (ΔN343)*, we used *Chimera 1* as template and primer-pair OMKB99 (BamHI site)-OMKB85 (PstI site) to amplify a 2,601 bp fragment corresponding to the *PfalMRE11* nuclease domain only. Next, we created a point mutation (*D398N*) in the nuclease domain of *PfalMRE11* using a splice overlap extension technique with *Chimera* 1 as template and the overlapping primers OMKB299 and OMKB298, to generate the construct *Chimera 1 (D398N)*. We also constructed another chimera in which the nuclease domain of ScMre11 (256 amino acids) was fused to the C-terminal domain of PfalMre11 (406 amino acids), creating *Chimera 2*. This was performed by amplifying 768 bp of N-terminal *SCMRE11* with the primer-pair OMKB84 (BamHI)-OMKB154 (HindIII), amplifying 1,218 base pairs of C-terminal *PfalMRE11* with the primer-pair OMKB153 (HindIII), OMKB89 (PstI), fusing the PCR two products and cloning the resulting fragment into a pTA vector. *Scmre11(D56N)* was PCR amplified from genomic DNA isolated from the yeast strain MKB4 using primer-pair OMKB84-OMKB85 and the resulting fragment was cloned into the pTA vector.

To generate a Mre11 plasmid for antibody production, we expressed the C-terminal domain of PfalMre11 in bacteria. This was done by amplifying a 1,131 bp fragment representing the C-terminus of *PfalMRE11* using the primer pair OMKB188 (BamHI)-OMKB166 (SalI), and

cloning the product into the pET28a vector (Novagen). All the recombinant plasmids were confirmed by DNA sequencing.

To create pRS413-kanMX6 plasmid for end joining assays, the kanMX6 cassette was excised from pFA6a-kanMX6 by Not I digestion and cloned into the Not I site of the plasmid pRS413.

For yeast two hybrid analysis, full length *PfalMRE11* was subcloned into the bait vector pGBDU-C1 and prey vector pGAD-C1 [34] to generate *PfalMRE11*-BD and *PfalMRE11*-AD, respectively. To study the interaction between PfalMre11 with ScXrs2 or ScRad50, *ScXRS2* and *ScRAD50* were cloned individually into the bait vector. *ScXRS2* was amplified using *S. cerevisiae* genomic DNA and the forward-reverse primer pair OMKB262-OMKB263, both of which had BamHI flanking sequences, and the PCR product cloned into a bait vector with an N-terminal *GAL4* DNA binding domain, generating an *ScXRS2*-BD fusion. Similarly, an *ScRAD50*-BD fusion was created by amplification of *ScRAD50* from *S. cerevisiae* genomic DNA with the primer pair OMKB164 (BamHI), OMKB165 (EcoRI) and cloning of the PCR fragment into the bait vector. *Chimera 1* was subcloned into a prey vector with an N-terminal *GAL4* activation domain to generate the *Chimera1*-AD fusion. The yeast *KU80* ORF was amplified from *S. cerevisiae* genomic DNA using OMKB210 (BamHI)-OMKB75 (SalI) primers and cloned into the pGBDUC1 vector. The *ScMRE11* gene was excised from the pTA vector and sub-cloned into the BamHI and PstI sites of the bait and the prey vectors. The *PfRAD50* ORF was PCR amplified from *P. falciparum* 3D7 genomic DNA using OMKB167 (EcoRI) and OMKB326 (PstI) primers and cloned into pGBDUC1 vector.

Yeast strains

Genotypes of the yeast strains used in this study are given in S2 Table.

The yeast expression vectors harboring *PfalMRE11*, *ScMRE11*, *ScMRE11C*, *Chimera 1*, *Chimera 1 (ΔN343)*, *Chimera 1 (D398N)* and *Chimera 2* were transformed into the *Δmre11* strain, MKB7, to generate the strains BSB3, BSB2, BSB6, BSB4, BSB7, PVY1 and BSB5, respectively. The empty pTA vector was transformed in W303α and MKB7 to generate BSB1 and BSB8, respectively. Strain PJ69-4A was used to study yeast two hybrid interactions. Initially, all bait fusion constructs were transformed into PJ69-4A and the transformants were selected on media lacking uracil to generate the strains BSB15, BSB22 and SAN1. As a control, the empty bait vector pGBDU-C1 was transformed into PJ69-4A to generate BSB14. Next, we transformed the empty prey vector into each of the strains BSB14, BSB15, BSB22 and SAN1 to generate BSB18, BSB20, BSB23 and SAN2, respectively. Similarly a prey-*PfalMRE11* fusion construct was transformed into each of the strains BSB14, BSB15, BSB22 and SAN1, generating BSB19, BSB21, BSB25 and SAN3, respectively. Prey-*ScMRE11* fusion construct was transformed into BSB22 and SAN1 to generate BSB24 and SAN4, respectively. Finally, the prey-*Chimera 1* fusion construct was transformed into BSB22 and SAN1 to generate BSB26 and SAN5, respectively. Strains SAN7-SAN10, BSB28, BSB30, BSB32 and BSB33 were also created in the similar fashion. Transformants carrying both the bait and prey fusion constructs were selected using media lacking uracil and leucine.

Yeast two hybrid analysis

Bait plasmids that were transformed into PJ69-4A were checked for self activation by plating on media lacking uracil and adenine; lack of growth ensured that the bait fusions did not lead to self activation. We performed yeast two hybrid analysis according to the published protocol [9]. Briefly, we analyzed the interactions between bait and prey fusion constructs by checking the growth of each of the strains BSB18-BSB21, BSB23-26, SAN2, SAN3 and SAN5 on media

lacking uracil, leucine and adenine as well as on media lacking uracil, leucine and histidine. Growth on plates containing these media was scored after incubation for 5 days at 30°C.

PfalMre11 protein expression and antibody generation

An N terminal His_6 tag pET28a:*PfalMRE11C* construct was transformed into *Escherichia coli* strain Rosetta (DE3). Expression of the recombinant protein was performed as described [35]. Briefly, cells were grown in LB media containing chloramphenicol and kanamycin at 37°C until OD_{600} 0.8. Recombinant protein expression was induced by addition of 1 mM IPTG and incubated for 4 h at 37°C. PfalMre11C protein expression was visualized by running the bacterial lysates on a 10% SDS-PAGE gel. The protein band corresponding to PfalMre11 was excised from the gel and used for immunizing rabbits for antibody generation.

Antibodies and Western blot analysis

The anti-His antibody (Santa cruz Biotechnology Inc., CA) and HRP-conjugated anti-rabbit secondary antibody (Promega) were used at 1:5,000 and 1:10,000 dilutions, respectively. The primary antibody against PfalMre11 was generated in rabbit and used at 1:4,000 dilutions, and HRP-conjugated anti-rabbit secondary antibody (Promega) was used at 1:12,000 dilutions. As a control, we used anti-PfHsp70 antibody (kindly provided by Dr. Nirbhay Kumar, Tulane University). We used a PfHsp70 primary antibody at 1:1,000 dilutions and anti-mouse secondary antibody (Promega) at 1:10,000 dilutions. Western blotting was performed as previously described [9]. Proteins were visualized by an enhanced chemiluminescence system (Pierce) and the band intensities were quantified by ImageJ software.

RNA isolation and RT-PCR

P. falciparum 3D7 culture having 10% parasitemia was synchronized using 5% sorbitol followed by harvesting at the ring, trophozoite and schizont stages were harvested. Total RNA was isolated from each stage specific culture with and without MMS treatment using the protocol as described [36]. Similarly, total RNA was isolated from yeast strains BSB1-BSB8 and PVY1 after incubation at 30°C using the acid-phenol method as described [33]. Equal amounts of RNA as measured by spectroscopic analysis (JASCO spectrophotometer EMC-709) were subjected to DNase I (Fermentas) digestion to remove contaminating genomic DNA. The absence of genomic DNA was confirmed by PCR prior to the reverse transcription reaction (S1 Fig). Synthesis of cDNA was performed as described [37]. Briefly, ~10 μg of total RNA was reverse transcribed using the reverse transcriptase (Qiagen), and then the cDNA product was subjected to semi-quantitative RT-PCR. Next, the cDNA product was diluted 1:50 and subjected to real time PCR using a SYBR-Green kit (Roche) as described [34] and the Applied Biosystems 7500 Fast Real Time PCR system. The threshold cycle (C_T) value of *ARP* transcript of each sample was used to normalize the corresponding C_T values of *PfalMRE11* transcripts. The normalized C_T values of *PfalMRE11* from different samples were compared to each other to obtain ΔC_T values. The relative levels of mRNA were deduced from the formula (Change in mRNA level = $2^{\Delta CT}$). The mean values (±SD) from three independent experiments were plotted using Graph Pad Prism 6 software. To quantify expression of wild-type and chimeric constructs from the yeast GPD promoter, the C_T values were normalized with the C_T value of *ScACT1* transcript. All the primers used in the semi-quantitative RT-PCR or real-time RT-PCR are presented in S1 Table.

MMS sensitivity assay

MMS sensitivity assays were performed as described [34]. Yeast strains BSB1-BSB8 and PVY1 were incubated in media lacking tryptophan at 30°C to OD_{600} 0.8. Half of each culture was spotted on Sc-Trp media containing 0.005% MMS, while the other half of each culture was spotted on Sc-Trp plates without MMS as a control. The plates were incubated for at 30°C for 72 hrs and their growth was compared. Similar experiments were also carried out using YPD media with 0%, 0.005% MMS, or 0.01% MMS.

To quantify the differences in MMS sensitivity of these strains, equal numbers of cells from rapidly dividing cultures were divided them into two groups: one group was treated with 0.005% MMS while the other was untreated. Both groups were incubated for 2 hours at 30°C after which MMS was washed out. Then, equal numbers of MMS-treated and untreated cells were spread onto media lacking tryptophan and incubated at 30°C for 72 hours. To determine the % survival, the ratio of the number of cells grown on MMS to the number of cells grown in the absence of MMS was calculate and multiplied by 100. Each assay was repeated a minimum of 3 times.

Plasmid end joining assay

Plasmid pRS413-kanMX6 was digested with XhoI and 400 ng of the resulting kanMX6 fragment linear (cut) or 400 ng of the undigested plasmid was transformed into each strain. The ratios of G418-sulphate resistant transformants were calculated for each strain. The assays were repeated thrice and the mean value ± SD was plotted using Graph Pad Prism 6 software.

Homology modeling

The model of PfalMre11 was obtained by submitting the full-length sequence of PfalMre11 to I-TASSER (Iterative Threading ASSEmbly Refinement) Web Server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). I-TASSER generates full-length models by a combination of Homology Modeling and Ab-Initio Modeling. For PfalMre11, the system identified and used the 4FBK and 3T1I PDB protein structures as templates that aligned primarily to the nuclease domain of Mre11. The unaligned regions were modeled using Ab-Initio modeling algorithm. We restricted our analysis to the nuclease domain of PfalMre11. Protein structures were derived from the highest confidence model generated by I-TASSER. The PyMOL Molecular Visualization Tool was used to visualize, analyze and generate the images.

Results

Identification and primary structure analysis of a Mre11 ortholog in *P. falciparum*

A Blast search of PlasmoDB revealed a single copy of the *PfalMRE11* gene in the *P. falciparum* 3D7 strain (Gene ID: PF3D7_0107800). The single exon *PfalMRE11* open reading frame (ORF) of 3,699 bp was PCR amplified from genomic DNA. The deduced amino-acid sequence of the PCR product was a 1,233 amino-acid long protein with a molecular mass of 146,149 Da and pI of 5.35. Homology searches of the PfalMre11 protein sequence revealed 14% to 22% overall similarity with other Mre11 orthologs. The PfalMre11 nuclease domain was 33% to 35% identical to the nuclease domains of other Mre11 orthologs (Table 1). Compared to Mre11 orthologs, PfalMre11 had the longest ORF (Fig 1A) and was 80% longer than average (1233 amino acids compared to an average length of 700 amino acids of other Mre11 orthologs). A genome database search of *Toxoplasma gondii*, another apicomplexan parasite,

Table 1. Homology of PfalMre11 with other eukaryotic Mre11 proteins.

	ScMre11	PfalMre11	TgMre11	TbMre11	AtMre11	DmMre11	MmMre11	HsMre11
ScMre11	100	23 (32)	31 (41)	26 (43)	30 (45)	32 (44)	34 (49)	34 (50)
PfalMre11		100	14 (34)	17 (35)	22 (34)	23 (34)	21 (36)	22 (37)
TgMre11			100	27 (45)	32 (42)	31 (43)	35 (47)	35 (46)
TbMre11				100	29 (49)	30 (49)	29 (49)	30 (50)
AtMre11					100	36 (50)	35 (56)	36 (56)
DmMre11						100	41 (57)	39 (56)
MmMre11							100	89 (96)
HsMre11								100

Numbers in parentheses represent homology within the nuclease domain. Sc, *S. cerevisiae*, Pfal, *P. falciparum*, Tg, *Toxoplasma gondii*, Tb, *Trypanosoma brucei*, At, *Arabidopsis thaliana*, Dm, *Drosophila melanogaster*, Mm, *Mus musculus*, Hs, *Homo sapiens*.

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revealed a putative TgMre11 ortholog that was also significantly longer (1244 amino acids) than other Mre11 orthologs (Fig 1A).

All eukaryotic Mre11 orthologs contain four highly conserved phosphodiesterase motifs in the N-terminal nuclease domain, and the first three are conserved in the EcSbcD, the Mre11 ortholog in *E. coli*. Multiple sequence alignments revealed that PfalMre11 also contains the four phosphodiesterase motifs (Fig 1B). Unlike the other Mre11 orthologs, PfalMre11 has a 350 amino acids N-terminal extension before the nuclease domain that extends from amino acid 351–796 (Fig 1A). Such a long N-terminal extension has not been observed in other Mre11 orthologs, with the exception of *T. gondii* TgMre11 which has a 220 amino acids N-terminal extension preceding its nuclease domain. The significance of such N-terminal extensions is currently unknown. In yeast Mre11, amino acids that are critical for nuclease function have been identified all four phosphodiesterase motifs [38]. These residues, D16, D56, H125 and H213 of ScMre11) are evolutionary conserved, including in PfalMre11 (D358, D398, H706 and H792) (Fig 1C). In addition, the spacing between the phosphodiesterase motifs is tentatively conserved among all Mre11 orthologs. The spacing between the 1st and the 2nd, between the 2nd and the 3rd, and between the 3rd and the 4th motifs range from 33–40 amino acids, 69–76 amino acids and 87–99 amino acids, respectively. In PfalMre11 this level of spacing is conserved only between the 1st and the 2nd (40 amino acids) and between the 3rd and the 4th motifs (86 amino acids). There is a large insertion of a low complexity region between the 2nd and the 3rd motifs (308 amino acids) that is not present in other parasitic Mre11 orthologs (i.e., TgMre11 or TbMre11). The significance of such an unusual structural feature within the PfalMre11 nuclease domain remains to be determined.

Mre11 contains two DNA binding domains (DBD): DBD-A is rich in basic amino acids, while DBD-B is rich in acidic amino acids [39, 40]. Although PfalMre11 as well as TgMre11 contain DBD-A, they do not contain DBD-B (Fig 1A). This is consistent with the results of mutational studies in yeast that identified DBD-A as being essential and DBD-B as dispensable for *in vivo* ScMre11 function [40]. At this time, it is difficult to assign any evolutionary significance to the absence of DBD-B in PfalMre11 or TgMre11.

The C-terminal domain of Mre11 plays a crucial role during DDR signaling [18], a function that is conserved in the C-terminal domains of yeast and mammalian Mre11 despite the poor sequence conservation at the amino acid level [41, 42]. The C-terminal domain of PfalMre11 is highly divergent from other Mre11 orthologs, making it impossible to predict whether PfalMre11 also possesses DDR signaling function. Thirteen amino acids, α-helical region specific to Mre11, known as the A470 motif, was first identified in yeast and later found to be

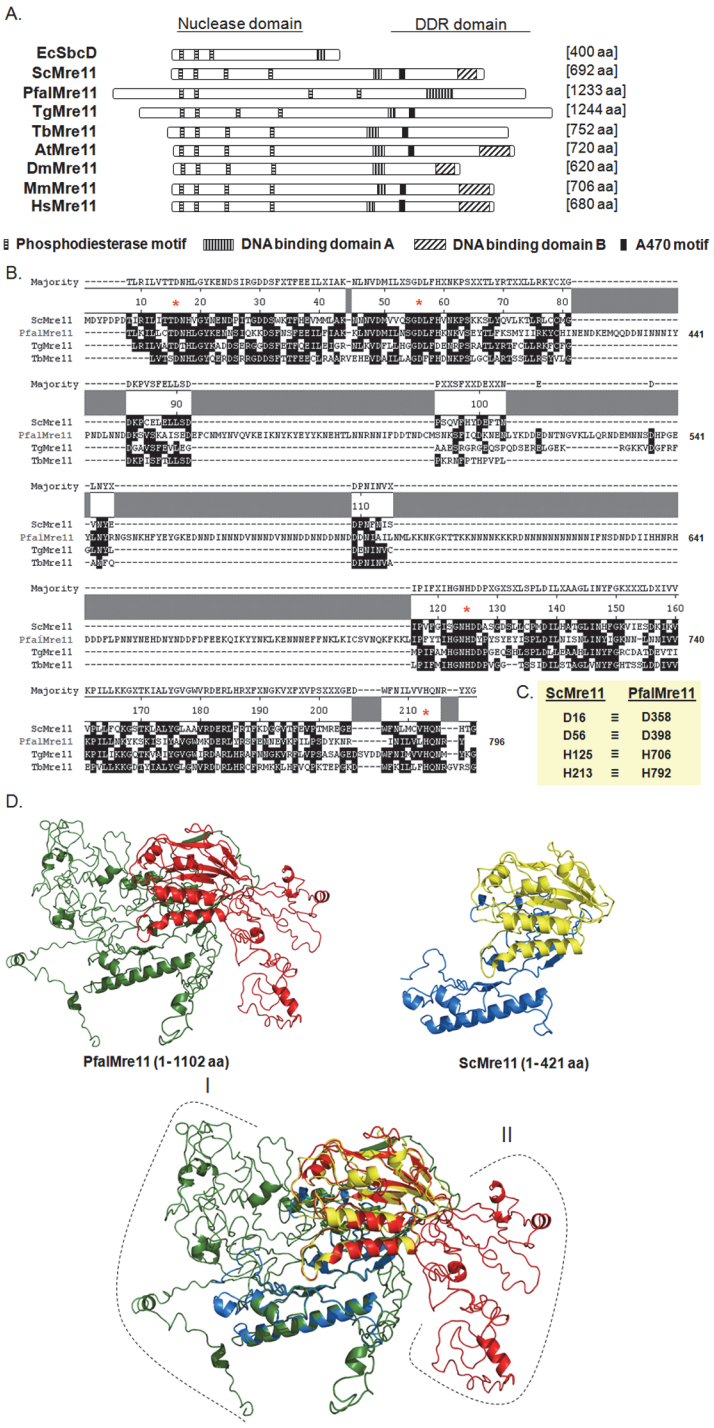

Fig 1. Primary structure analysis of PfalMre11. (A). Schematic representation of different domains and motifs of PfalMre11 in comparison with other Mre11 proteins from bacteria (EcSbcD); yeast (ScMre11); *Toxoplasma gondii* (TgMre11); *Trypanosoma brucei* (TbMre11); *Arabidopsis thaliana* (AtMre11); *Drosophila melanogaster* (DmMre11); mouse (MmMre11) and human (HsMre11). The four phosphodiesterase motifs within the nuclease domain are not only conserved among all the eukaryotic Mre11 but also are present in *E. coli* SbcD nuclease protein. PfalMre11 lacks both DNA binding domain-B and A470 motif (AV*(E/K)FV(E/D) K(D/E)(D/E)K*A, where the asterisk refers to sites that accommodate multiple residues). (B) Multiple sequence alignment showing sequence conservation within the nuclease domain of PfalMre11 with ScMre11 and other parasitic Mre11 proteins (TgMre11 and TbMre11). The functionally critical amino-acid residues in each phosphodiesterase motifs (namely, D358, D398, H706 and H792) are marked by red asterisk. The

coordinates of the amino-acids positions of ScMre11 is given on the top and that of the PfalMre11 is given on the right. (C) The four critical amino-acid residues of each of the phosphodiesterase motif of ScMre11 (D16, D56, H125 and H213) and the corresponding amino-acids of PfalMre11 are shown. (D) Predicted three-dimensional structure of PfalMre11 N-terminal domain. Homology models of PfalMre11 N-terminal region (amino-acids 1–1102) and ScMre11 N-terminal region (amino-acids 1–421) are shown. The nuclease domain of PfalMre11 is shown in red, while the rest of the N-terminal is shown in green. Similarly, the nuclease domain of ScMre11 is shown in yellow, while the rest of the N-terminal is shown in marine blue. The long N-terminal extension (region I: amino-acids 1–349) and several stretches of insertions within the nuclease domain (region II) of PfalMre11 are also indicated.

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conserved in other eukaryotes, with the exception of dipterans [30]. This motif plays regulatory roles in telomere recombination and in telomere rapid deletion [30]. Surprisingly, this motif is missing from PfalMre11, but present in protozoan TgMre11 and TbMre11 (S2 Fig).

Comparison of the predicted three dimensional structures of the N-terminal regions of PfalMre11 and ScMre11 by homology modeling revealed striking structural similarity in their nuclease domains despite having a relatively low level of sequence identity (32%). Indeed, it is a near perfect superimposition of the core nuclease domains. The superimposed image revealed that the long N-terminal extension (region I) and the low complexity insertion (region II) protrude out of the core structure (Fig 1D).

Expression of recombinant PfalMre11 protein and generation of antibody

The *PfalMRE11* ORF was cloned into three bacterial expression vectors (pET28a, pGEX and pMALC2) and the recombinant plasmids were each transformed into three strains of *E. coli* (namely, BLl21 DE3, Rosetta DE3 and BL21 pLysS DE3). Western blot analyses using anti-His antibody confirmed that recombinant PfalMre11 was not expressed (data not shown). The PfalMre11 N-terminus (PfalMre11-N: 832 amino acids) and the PfalMre11 C-terminus (PfalMre11-C: 377 amino acids) were separately cloned into the pET28a vector and transformed into the *E. coli* strains described above. Only the expression of PfalMre11-C protein was observed in the Rosetta DE3 strain (having the pRARE plasmid that supplies tRNAs for six rare amino acids). S3A Fig (lane 5) shows that recombinant PfalMre11-C corresponds to about 15% of the total protein in the crude cell extract after IPTG induction. Expression of the recombinant protein was confirmed by Western blot analysis (S3B Fig). Because the induced recombinant protein was predominantly expressed in the insoluble fraction and could not be purified; therefore, the induced protein band was excised from the SDS-PAGE gel, purified and and injected into rabbits for antibody production. Rabbit anti- PfalMre11 antibody reacted positively with recombinant PfalMre11-C protein expressed in bacteria, confirming the specificity of the antibody (S3C Fig). When tested with parasite lysate, the anti-PfalMre11 antibody specifically recognized a protein band near 145 kDa that was not detected by pre-immune sera (S3D Fig). The size of the band corresponded to the predicted molecular mass of the PfalMre11 protein (146 kDa).

Developmentally regulated expression of PfalMre11 during blood stage development

To examine the expression of *PfalMRE11* during the intra-erythrocytic growth of *P. falciparum*, we synchronized parasites at the ring, trophozoite and schizont stages and performed RT-PCR and Western blot analysis. Semi-quantitative RT-PCR data showed that the steady-state levels of the *PfalMER11* transcripts were more abundant in the trophozoite and schizont stages than in the ring stage (Fig 2A). Expression of another parasitic gene *PfARP* (Asparagine

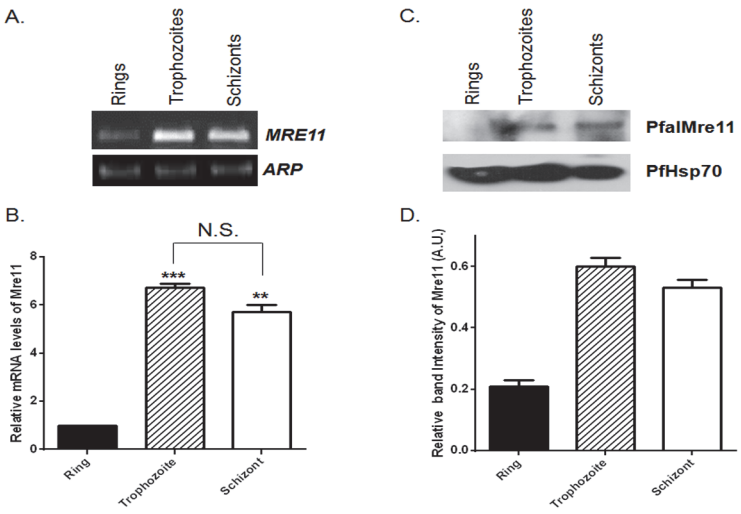

Fig 2. Developmentally regulated expression of PfalMre11 during blood stage development. (A). Semi-quantitative RT-PCR showing expression of *PfalMRE11* mRNA at the ring, trophozoite and schizont stages. *PfARP* was used as the loading control. (B). Relative abundance of *PfalMRE11* transcript measured by real-time RT-PCR analysis. Data were normalized against *PfARP*. The mean values ± SD from three independent experiments are plotted. (C). Stage specific expression of PfalMre11 protein. The stages are marked on the top. PfHsp70 was used as the loading control. (D). The quantification of Western blots from three independent experiments. Data was normalized against the loading control PfHsp70. Each bar represents mean density ± SD. The *P* value was calculated using the two-tailed Student's t-test (** means $P < 0.01$; *** means $P < 0.001$; N.S. means not significant).

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Rich Protein) remained unchanged throughout intra-erythrocytic development and hence was used as the loading control. To more accurately estimate levels of *PfalMRE11* transcripts during the three growth stages, we performed real-time RT-PCR and normalized the data with the expression levels of the *PfARP* transcripts. Compared to the ring stage, the PfalMRE11 transcript was 7 times more abundant in the trophozoite stage and 5 times more abundant in schizont stage (Fig 2B). Stage-specific expression of PfalMre11 protein was consistent with the RT-PCR results (Fig 2C). PfHsp70 protein levels remained stable during all three parasitic developmental stages and served as the loading control. Quantification of the band intensities revealed that the steady-state levels of PfalMre11 protein were 3 times and 2.5 times more abundant in the trophozoite and schizont stages, respectively, than in the ring stage (Fig 2D). Thus, the stage-specific changes in expression of PfalMRE11 at the RNA and the protein levels demonstrate that it is developmentally regulated and that such regulation is more likely to occur at the level of transcription rather than translation.

DNA damage- induced up-regulation of PfalMre11 during intra-erythrocytic development

Previous studies have shown that *MRE11* expression isup-regulated in response to DNA damage in a variety of organisms [43–45]. Mre11 is a "first responder" to DSBs where it activates the DDR pathway and recruits various effector molecules involved in DNA repair mechanisms [46]. We sought to investigate: (a) whether *PfalMRE11* is up-regulated in response to genome-wide DNA damage caused by MMS, and (b) whether such up-regulation is developmentally regulated. To this end, we synchronized *P. falciparum* at the ring, trophozoite and schizont stages, treated the cultures with MMS, and compared *PfalMRE11* transcript levels in both treated and untreated cultures using semi-quantitative RT-PCR and real-time RT-PCR analyses.

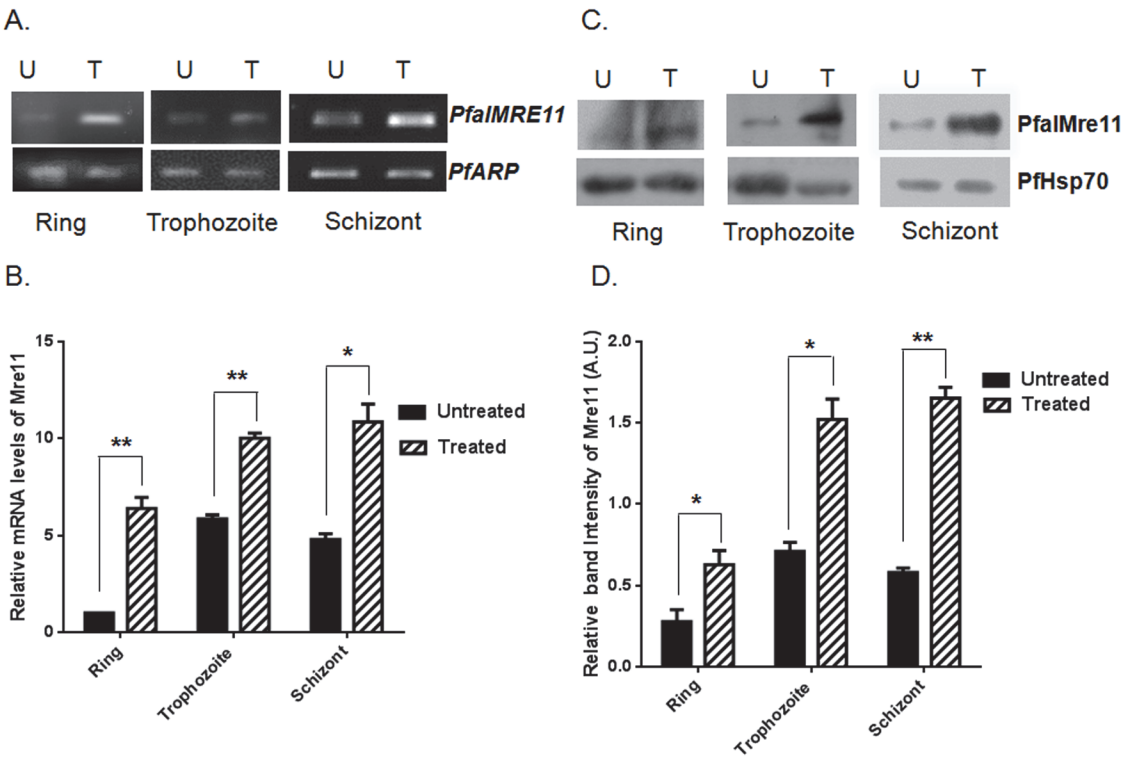

Fig 3. DNA damage induced up-regulation of PfalMre11 during intra-erythrocytic development. (A) *P. falciparum in vitro* cultures were synchronized at the ring, trophozoite or schizont stage and then either un-treated (U) or treated with 0.05% MMS (T) for six hours. Semi-quantitative RT-PCR from extracted RNA revealed up-regulation of *PfalMRE11* mRNA at all stages. *PfARP* transcript was used as the loading control. (B). Real-time RT-PCR data showing fold up-regulation of *PfalMRE11* mRNA at the ring, trophozoite and schizont stage, respectively. Data were normalized with the abundance of *PfARP* transcript. Each bar represents mean value ± SD from three independent experiments. (C). Western blots showing MMS induced expression of PfalMre11 protein at the ring, trophozoite and schizont stages. U: untreated; T: treated with MMS. PfHsp70 acted as the loading control. (D). The quantification of Western blots exhibiting fold induction of PfalMre11 at the ring, trophozoite and schizont stages. Data were normalized against the loading control PfHsp70. Each bar represents mean density ± SD from three independent experiments. The *P* value was calculated using the two-tailed Student's t-test (* means $P < 0.05$; and ** means $P < 0.01$).

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PfalMRE11 transcripts were up-regulated in response to MMS treatment at all three developmental stages (Fig 3A). Real-time RT-PCR data revealed that upon DNA damage, *PfalMRE11* expression was 6-fold greater at the ring stage, and 2-fold greater at the trophozoite and schizont stages, than *PfalMRE11* expression in untreated cultures (Fig 3B). Equal amounts of total RNA from untreated and treated cultures were used in these experiments. The expression of house-keeping gene *PfARP* remained unaffected upon MMS treatment and hence it is used as the normalization control.

Next, PfalMre11 protein expression was examined in cultures that were synchronized at the ring, trophozite and schizont stages and treated with MMS. At 6h of MMS treatment, expression of PfalMre11 was markedly increased compared to untreated cultures at all three developmental stages (Fig 3C). Quantification of the band intensities showed nearly 2-fold increase in PfalMre11 expression at all the three stages (Fig 3D).

Functional complementation of a *Δmre11* mutant of *S. cerevisiae* by PfalMre11 nuclease domain in conjunction with the DDR domain of ScMre11

To confirm *PfalMRE11* as a bona fide *MRE11* ortholog, we performed a functional complementation assay to determine if *PfalMRE11* could functionally complement a yeast *Δmre11* null mutant. The strains BSB3, BSB2 and BSB8 were constructed by transforming pTA*PfalMRE11*, pTA*ScMRE11* and pEmpty plasmids into the *Δmre11* strain, respectively. Since *Δmre11* cells are hyper-sensitive to MMS, a rescue of the MMS sensitivity phenotype would indicate complementation of Mre11 function. We observed no functional complementation of *Δmre11* by *PfalMRE11* (data not shown).

ScMre11 has two distinct activities that are required for repair of DSB: the N-terminally confined nuclease activity and the C-terminally located DDR activity. As demonstrated above, the N-terminus of PfalMre11 shares considerable sequence identity with other Mre11 orthologs, and the PfalMre11 nuclease domain shares structural similarity with ScMre11. On the other hand, the C-terminus of PfalMre11 is highly divergent compared to other Mre11 orthologs and shares limited sequence similarity with ScMre11. Therefore, we hypothesized that PfalMre11 possessed N-terminal nuclease activity, and the C-terminal domain could not interact with molecules involved in the yeast DDR pathway. To test this hypothesis, we constructed several complementation vectors carrying recombinant genes (Fig 4A) and transformed then independently into a yeast *Δmre11* strain to determine if the N-terminus or C-terminus of PfalMre11 could complement Mre11 function. Chimera 1 consisted of the N-terminal region of PfalMre11 (amino acids 1–832) and the C-terminal region of ScMre11 (amino acids 315–692); Chimera 2 consisted of the N-terminal domain of ScMre11 (amino acids 1–256) and the C-terminal region of PfalMre11 (amino acids 727–1132). Another complementation vector was constructed with the C-terminal region of ScMre11 alone. These regions of *PfalMRE11* and *ScMRE11* genes were selected based on homology analysis of the nuclease domain. Expression levels of the hybrid genes were confirmed by real-time RT-PCR (Fig 4B); however, the proteins could not be detected by Western blot analysis using anti-Myc antibody, possibly due to low expression levels.

Next, *Δmre11* strains harboring different complementation plasmids were tested for functional complementation analysis. We examined MMS sensitivity using a spotting assay and a return-to-growth assay. In the spotting assay, dilutions of different strains were spotted on MMS containing plates and allowed to grow for several days. Thus, the cells were continuously exposed to MMS for the entire growth period. On the other hand, in the return-to-growth assay, the cells were exposed to MMS for two hours and then returned to grow on plates lacking MMS. Thus, the first experiment tests the cells' ability to survive when challenged with continuous DNA insult and the second experiment measures the cells' ability to repair DNA damage after a brief exposure to MMS. Chimera 1 rescued MMS sensitivity of *Δmre11* which is comparable to ScMre11 (Fig 5A and S4 Fig). Chimera 2 remained as hyper-sensitive to MMS as the *Δmre11* null strain (Fig 5A). Since Chimera 1 contained the C-terminal domain of ScMre11, we also tested the possibility that the reversal of MMS sensitivity was not due solely to the C-terminal domain of ScMre11. When the C-terminal domain of ScMre11 was expressed alone, we found no reversal of MMS sensitivity (Fig 5A). Taken together, these results support the hypothesis that the N-terminus of PfalMre11 has nuclease function, but the C-terminus lacks the DDR function ascribed to ScMre11.

To gain further insights into the structure-function relationship of the PfalMre11 N-terminal domain, we generated two mutants of Chimera 1: a missense mutant (D398N) and a deletion mutant (Δ1–343). It was previously reported that aspartic acid (D) at the 56th position of

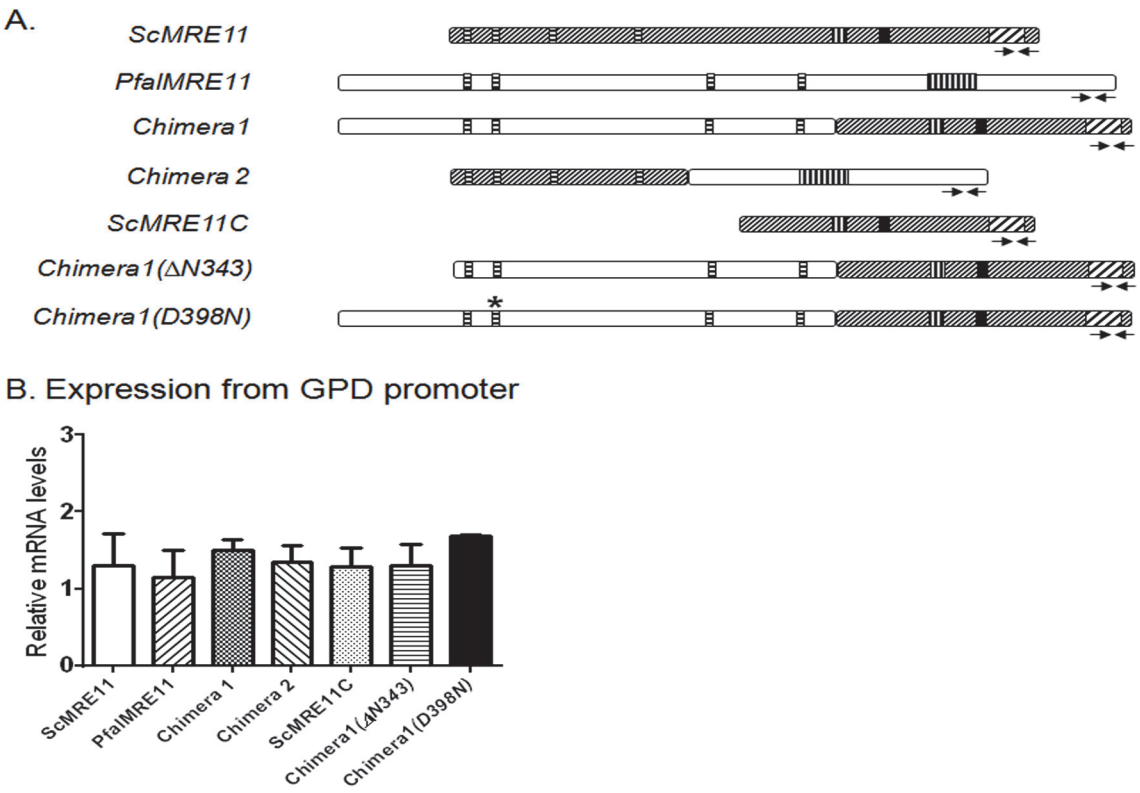

Fig 4. Expression levels of different constructs used in functional complementation. (A). Schematic representations of genes or gene fragments used in the complementation experiments. ScMre11: full length *MRE11* gene from *S. cerevisiae*. *PfalMRE11*: full length *MRE11* gene from *P. falciparum*. Chimera 1: chimeric gene consisting of DNA sequences corresponding to the N-terminal of PfalMre11 (1–832 amino acids) fused to the DNA sequence corresponding to the C-terminal domain of ScMre11 (315–692 amino-acids). Chimera 2: chimeric gene consisting of DNA sequences corresponding to the N-terminal of ScMre11 (1–256 amino acids) fused to the DNA sequence corresponding to the C-terminal domain of PfalMre11 (828–1233 amino-acids). *ScMRE11C*: gene fragment corresponding to the C-terminal domain of ScMre11 (315–692 amino-acids). Chimera 1(N343): chimeric gene consisting of DNA sequences corresponding to PfalMre11 (344–832 amino acids) fused to the DNA sequence corresponding to the C-terminal domain of ScMre11 (315–692 amino-acids). Chimera 1 (D398N): same as chimera 1 with a missense mutation (D to N) at the 398th amino-acid of PfalMre11. (B). Real-time RT-PCR data showing comparable expression of each gene or gene fragment used in the complementation assay. In each case mean value (± SD) from three different experiments is normalized against the abundance of *ACT1* mRNA.

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ScMre11 lies in the second phosphodiesterase motif and is critical for nuclease activity [47]. Using site-directed mutagenesis, we created a corresponding amino acid substitution, D398N, in Chimera 1. When tested for functional complementation, this mutant exhibited *Δmre11* like MMS hyper-sensitivity (Fig 5A), suggesting that PfalMre11^{D398N} is also a nuclease-deficient mutant. Such a dramatic effect of D398N is surprising, given that the corresponding yeast mutant *mre11(D56N)* is only mildly sensitive to MMS (S4 Fig) [47]. As stated earlier, the PfalMre11 nuclease domain covers amino acids 350–796 and is preceded by an unstructured N-terminal extension which is absent in all other Mre11 orthologs. In order to understand whether this unusual N-terminal extension is dispensable for the N-terminal function of PfalMre11, we deleted the first 343 amino acids of Chimera 1 and tested the construct for MMS sensitivity in a *mre11* null yeast strain. This deletion mutant failed to complement loss of ScMre11 function to normal levels, suggesting that the entire N-terminal extension of PfalMre11 is indispensable for the N-terminal function of PfalMre11 (Fig 5A and S4 Fig). It is possible that only a section of this 343 amino acid stretch is indispensable; however, since this region does not contain any particular motif/structural elements, it is difficult to design smaller deletion mutants to test this possibility. The expression levels of both the D398N and N-

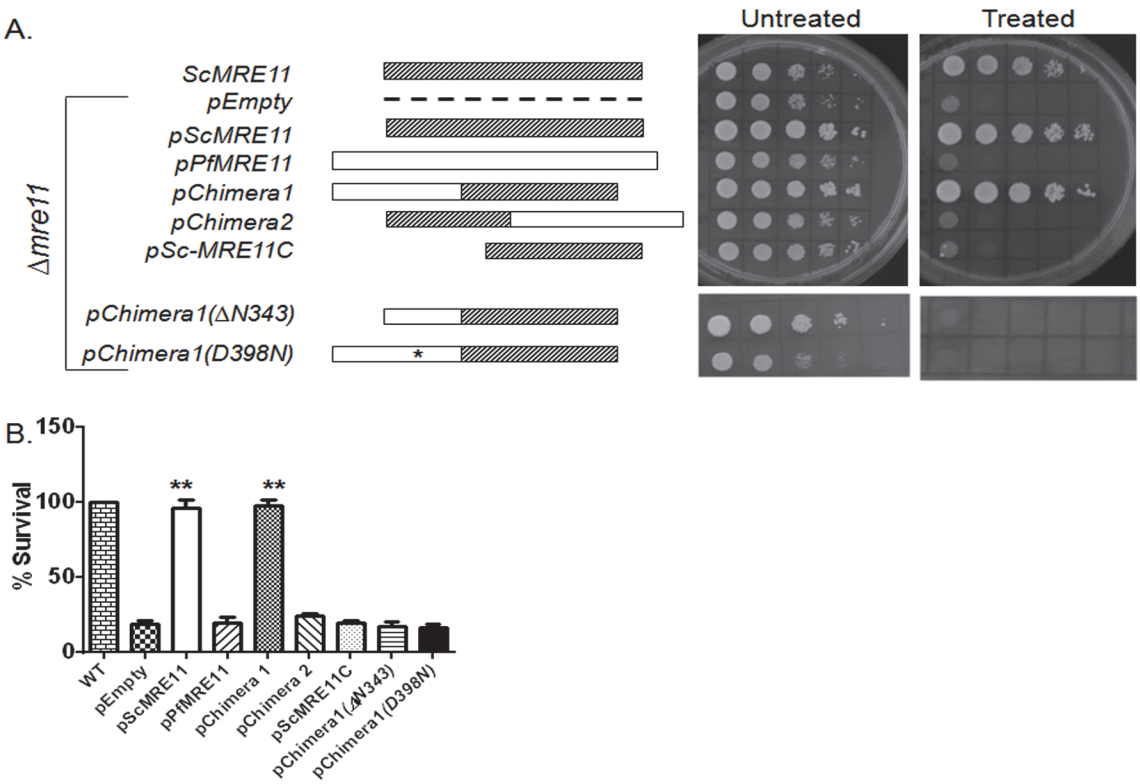

Fig 5. Functional complementation of *Δmre11* mutant of *S. cerevisiae* by PfalMre11 nuclease domain in conjunction with the DDR domain of ScMre11. (A). Spotting assay on media lacking tryptophan without (untreated) or with (treated) 0.005% MMS supplementation. The relevant genotypes along with the corresponding schematic diagrams are shown on the left. (B) Various complementation strains (as shown on the X-axis) were treated with 0.005% MMS for 2 hours and then returned to growth on media lacking tryptophan and MMS. The number of surviving colonies were scored and compared with the number arising from un-treated cells. Each bar represents the mean number ±SD after normalizing with untreated controls from four independent experiments. The *P* value was calculated by two-tailed Student's t-test (** indicates $P < 0.01$).

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terminal deletion mutants were tested by real-time RT-PCR analysis and mRNA expression of both of the mutants were on par with the mRNA expression of Chimera 1 (Fig 4B).

Return to growth experiments also revealed similar results. Chimera 1 complemented ScMre11 deficiency, while the phenotype observed with either PfalMre11 or Chimera 2 was indistinguishable from the *Δmre11* null strain (Fig 5B). This result suggests that the N-terminal domain of PfalMre11 in conjunction with the C-terminal domain of ScMre11 is capable of repairing MMS induced DNA breaks. Such repair activity was abrogated by Pfal D398N and PfalMre1-N-terminal (Δ343) also abrogated the repair activity of Chimera 1 (Fig 5B). Since the proteins could not be detected by Western blot, we cannot formally rule out the possibility that the lack of complementation is not due to reduced stability of the recombinant proteins.

PfalMre11 is capable of interacting with ScRad50 and ScXrs2

Mre11 acts as MRX complex in yeast, where two molecules of Mre11, two molecules of Rad50 and one molecule of Xrs2 form a hetero-pentameric complex. The demonstration that Chimera 1 (N-terminus of PfalMre11 fused to C-terminal of ScMre11) could sufficiently complement ScMre11 deficiency suggests that it interacts in a MRX complex consisting of Chimera1-ScRad50-ScXrs2. We used a yeast two hybrid system to test whether PfalMre11 (or Chimera 1) could interact with itself as well as ScRad50 and ScXrs2. A *GAL4* transcriptional activation system in PJ69-4A cells was used to distinguish robust and feeble interactions between bait and

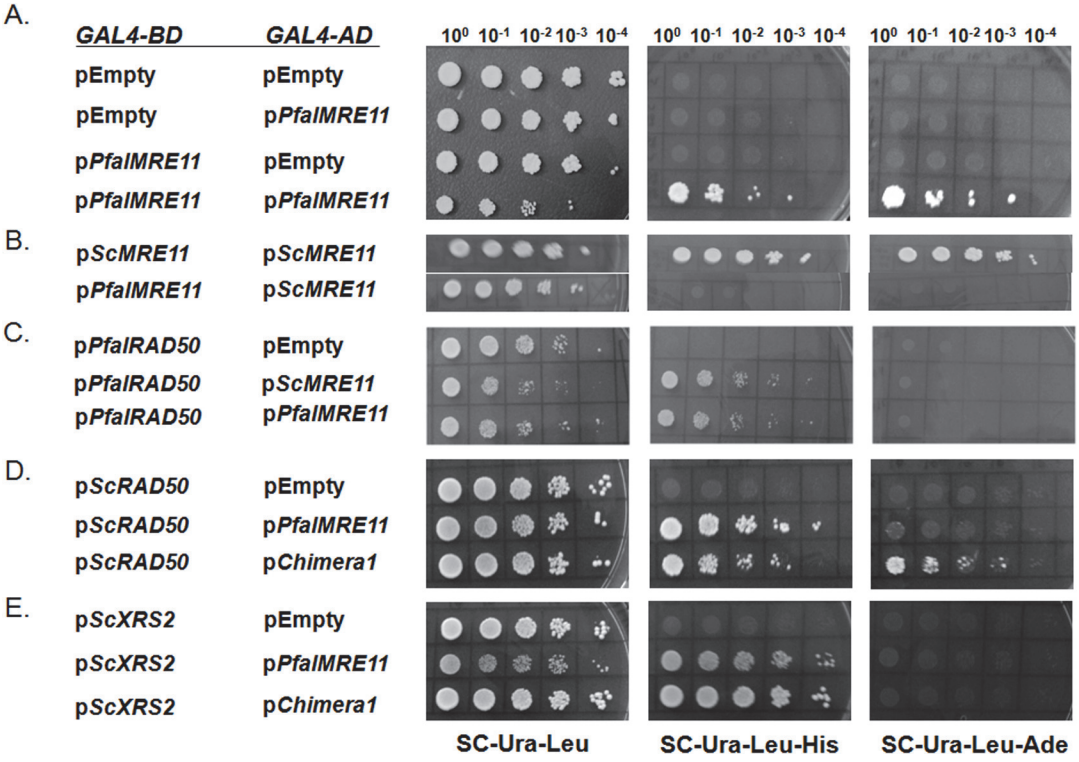

Fig 6. PfalMre11 is capable of forming MRX complex in yeast. (A) A DNA fragment corresponding to the full length *PfalMRE11* ORF was fused to the *GAL4* activation domain (*GAL4-AD*) in pGADC1 as well as to the *GAL 4* DNA binding domain (*GAL-BD*) in pGBDUC1. Two hybrid interactions were tested with yeast strain PJ694A, which carries *ADE2* and *HIS3* genes as reporters. Starting with the same OD (1 OD/ml), five fold serial dilutions were prepared and spotted on media lacking uracil and leucine (SC-Ura-Leu) as a control, as well as media lacking uracil, leucine and histidine (SC-Ura-Leu-His) or media lacking uracil, leucine and adenine (SC-Ura-Leu-Ade) to test for protein-protein interactions. (B) *ScMRE11* or *PfalMRE11* were cloned into pGBDUC1 to produce fusion protein with *GAL4* DNA binding domain. Interactions with *ScMRE11* were scored. (C) *PfalRAD50* were cloned into pGBDUC1 to produce fusion protein with *GAL4* DNA binding domain. Interactions with *ScMRE11* or *PfalMRE11* were scored. (D) *ScRAD50* was cloned into pGBDUC1 to produce a fusion protein with a *GAL4* DNA binding domain. Interactions with *PfalMRE11* or Chimera 1 were scored. (E) *ScXRS2* was fused to the DNA binding domain in pGBDUC1 and tested for interaction with *PfalMRE11* or Chimera 1.

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prey proteins; a robust interaction was indicated by growth on (Sc-Leu-Ura-Ade) triple drop-out plates and a weaker interaction was indicated by growth on (Sc-Leu-Ura-His) triple drop-out plates. We observed robust self-interaction of PfalMre11 protein (Fig 6A). These results suggest that PfalMre11 may act as a dimer, consistent with other eukaryotes. Interaction between full length PfalMre11 and full length ScMre11 was not observed (Fig 6B). We also observed physical interaction between PfalMre11 and PfalRad50, suggesting the existence of the MR complex in the parasite (Fig 6C). When tested for interaction with ScRad50, both PfalMre11 and Chimera 1 interacted; however, the interaction between PfalMre11 and ScRad50 was significantly weaker (Fig 6D). PfalMre11 as well as Chimera 1 also interacted with ScXrs2; however, these interactions were not robust given that growth was observed only on (Sc-Leu-Ura-His) triple dropout plates (Fig 6E). Taken together, these results suggest that PfalMre11 interacts with individual components of the MRX complex in the heterologous yeast system.

NHEJ activity of Chimera 1

Mre11 plays crucial role in NHEJ mediated DSB repair. Since our results demonstrated that PfalMre11 repairs MMS-induced DNA damage, and interacts with ScRad50 and ScXrs2, we

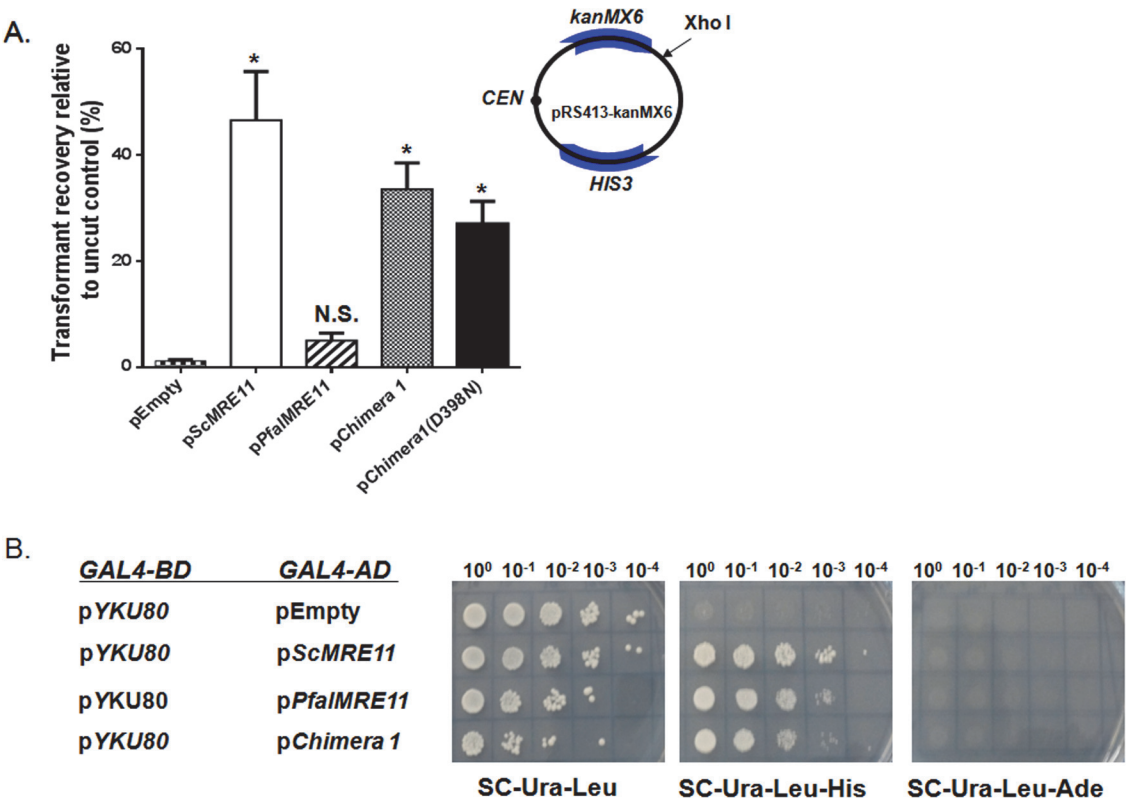

Fig 7. End joining activity of Chimera 1 consisting of PfalMre11 nuclease domain in conjunction with the DDR domain of ScMre11. (A) The relevant genotype of each strain is marked on the X-axis. The schematic diagram of the plasmid used for this assay is shown in the inset. The end joining assays were performed as described in the Method section. (B) PfalMre11 is capable of interacting with YKu80. Yeast-two- hybrid analysis between YKu80 cloned into the bait vector (pGBDUC1) vector and PfalMre11 or Chimera 1 cloned into the prey vector (pGADC1). ScMre11 acted as a positive control. The *P* value was calculated by two-tailed Student's t-test (* indicates $P < 0.05$, N.S. means not significant).

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wanted to investigate whether PfalMre11 acts in in DSB repair through NHEJ. To test this, we used a plasmid transformation assay in which the efficiency of the recircularization of a linearized plasmid was used as a measure of NHEJ (Fig 7A). Yeast strains harboring different complementation constructs were transformed with an equal amount of either linear plasmid or circular plasmid (control). The efficiency of NHEJ was measured as the ratio of the number of transformants obtained with linear plasmid to the number of transformants obtained with circular plasmid. Compared to the control, Chimera 1 showed moderate NHEJ efficiency compared to ScMre11 while the relatively low number of transformants with PfalMre11 indicated no significant NHEJ activity. The Chimera 1 (D398N) mutant did not resulted in abrogation of NHEJ activity (Fig 7A), which corroborates with the previous finding that the nuclease activity of ScMre11 is dispensable for NHEJ activity [47]. A large body of evidence has demonstrated that interaction between Mre11 and yKu80 is important for NHEJ [48]; therefore, we investigated whether Chimera 1 or PfalMre11 also interacts with yKu80. We observed that both Chimera 1 and PfalMre11 interacted with yKu80, although the interactions were not robust (Fig 7B). Since the Ku complex as well as NHEJ are apparently absent in *P. falciparum*, the physiological significance of these findings is currently uncertain.

Discussion

Here we provide several lines of evidence that show the ORF (Gene ID: PF3D7_0107800), described as a putative Mre11 ortholog in PlasmoDB, codes for a bona fide Mre11 protein in *P. falciparum*. Firstly, this protein is predominantly expressed during the mitotically active schizont stage of the parasite. Secondly, the expression of PfalMre11 is induced in response to DNA damage. Thirdly and most importantly, the nuclease domain of PfalMre11 can functionally complement the nuclease function of yeast Mre11. Fourthly, PfalMre11 can interact with PfalRad50. Finally, PfalMre11 is also capable of forming a complex with yeast Rad50 and Xrs2, two factors that are essential for Mre11 activity.

Although, the steady-state level of PfalMre11 was less abundant at the ring stage as compared to the trophozoite or the schizont stage, its up-regulation in response to DNA damage was observed at all three stages. This finding implies that the regulatory trans-factors that activate PfalMRE11 gene expression upon DNA damage are likely to be present at all stages during intra-erythrocytic development in the host. Given that the trophozoite and the schizont stages are metabolically more active and hence are likely to be more prone to DNA damaging free radicals, the question arises whether the increased abundance of PfalMre11 at these later stages is in response to spontaneous DNA damage as a result of exposure to free radicals or replication stress, or is purely developmentally regulated. Currently, the answers to such questions are unknown.

In eukaryotes, a DNA DSB is repaired either by the HR or NHEJ pathway. Mre11 plays a central role during the "decision-making" process that leads to either HR or NHEJ. Although *Plasmodium* apparently lacks NHEJ, it does possess the poorly defined A-NHEJ pathway [8]. It is not known whether PfalMre11 plays a similar role during the pathway choice of either HR or A-NHEJ. Given that *Plasmodium* has a haploid genome, a DNA DSB can be repaired by the HR mechanism only during or after the S phase (the trophozoite and the schizont stages). A DSB occurring in the G1 phase (the ring stage) is likely to be repaired by another mechanism, e.g. A-NHEJ. Currently, it is not known to what extent, if at all, A-NHEJ pathway is utilized in the ring stage of *P. falciparum*. Nonetheless, since Mre11 is common to all eukaryotic DSB repair pathways, it is not surprising that PfalMre11 can be found at all cell cycle phases in *Plasmodium*.

The DDR function of MRX is mediated through the interaction of Xrs2 (Nbs1) with Tel1 (the yeast ortholog of *ATM*) [49]. The lack of PfXrs2/Nbs1 and *PfATM* or *PfATR* in the *Plasmodium* genome sequences is puzzling. It is possible that *Plasmodium* possesses the structural and functional orthologs of these important DDR proteins but the amino acid sequences of these proteins may not be conserved. Our finding that PfalMre11 is capable of interacting with ScXrs2 supports this notion. Although, PfalRad50 has been annotated in the *Plasmodium* genome, earlier work on genome-wide yeast two hybrid analysis using a cDNA prey library of 400–500 bp average size failed to identify interaction between PfalMre11 and PfalRad50 [50]. In the current study, we are able to detect weak interaction between PfalMre11 and PfalRad50 using a full length genomic fragment of the *PfalRAD50* ORF. This finding once again suggests the conservation of PfalMre11 function.

Our results demonstrated that PfalMre11 failed to functionally complement ScMre11 in an *mre11* null mutant, whereas a fusion between the nuclease domain of PfalMre11 and the DDR domain of ScMre11 successfully complemented *Δmre11*. We also demonstrated that both the fusion protein (Chimera 1) and the PfalMre11 can form the MRX complex in yeast. Thus, we speculate that other important yeast proteins of the DDR pathway which interact with ScMre11 could not interact with full length PfalMre11, but they could interact with the Chimera 1 protein, resulting in functional complementation. Additional studies are needed to shed

light on the nature of these interactions. We observed that the Chimera 1 (D398N) nuclease mutant is defective in repairing MMS induced DNA damages but it could engage in plasmid end joining, suggesting that the nuclease activity of Chimera 1 is dispensable for NHEJ. Currently, we do not know why this mutant displayed such a strong MMS sensitive phenotype while the corresponding yeast mutant (D56N) is only mildly sensitive to MMS. It could be possible that this mutation affects the structure of Chimera 1 in such a way that the protein fails to engage itself with other DNA repair proteins involved in this pathway.

The 13 amino-acid alpha-helical region of ScMre11 (known as the A470 motif) is evolutionarily conserved from yeast to humans, with the exceptions of dipterans and, as reported here, *Plasmodium*. This motif is involved in several aspects of telomere maintenance, including telomerase-mediated elongation and telomere repeat recombination. Given that telomere maintenance in dipterans is transposition based, one possible interpretation for absence of the Mre11 A470 motif is because it is specific to the telomerase pathway and telomere repeat recombination. However, *P. falciparum* lacks the A470 motif although it is dependent on telomerase-mediated telomere addition. The role of PfalMre11 in telomere maintenance remains an open question.

It is well established that loss of Mre11 function in both yeast and humans causes sensitivity to DNA damage [51, 52]. Since Mre11 is involved in the upstream DDR pathway, which is common to all eukaryotic DSB repair mechanisms, it is likely that inactivation of PfalMre11 function could render the malaria parasites vulnerable to DNA damage. Since *Plasmodium* and human Mre11 proteins share only 22% sequence identity, it might be possible to specifically target the parasitic Mre11 with small molecule inhibitors.

Supporting Information

S1 Fig. (A) Minus RT-PCR on DNase treated RNA isolated from different yeast strains (as indicated on the top) shows absence of genomic DNA contamination in the samples. Primer pair OSB14 and OSB16 was used to amplify a 307 bp fragment of the *ACT1* gene/mRNA. PCR was performed for 30 cycles. (B) Minus RT-PCR on DNase treated RNA isolated from different stages *of P. falciparum in vitro* cultures shows absence of genomic DNA contamination. Primer pair OSB94, OSB95 was used to amplify a 300 bp fragment of the *PfARP* gene/mRNA. (C) Minus RT-PCR on DNase treated RNA isolated from different stages of untreated (control) or MMS treated (MMS) *P. falciparum in vitro* cultures showed absence of genomic DNA contamination. Primer pair OSB94, OSB95 was used to amplify a 300 bp fragment of the *PfARP* gene/ mRNA. M: DNA molecular weight marker (as marked on the left).
(TIF)

S2 Fig. Multiple sequence alignment reveals the lack of A470 motif in PfalMre11. The coordinate of ScMre11 and PfalMre11 amino-acid sequences are given on the left. The highly conserved amino-acid residues are highlighted in cyan and the semi-conserved residues are highlighted in pink.
(TIF)

S3 Fig. Expression of recombinant PfalMre11 protein and generation of antibody. (A). A polypeptide corresponding to the last 377 amino-acids of PfalMre11 (as indicated by the hatched box) was expressed as an N-terminal His-tagged protein (marked by an arrow). Expression profile from cell lysates taken at 0 hour (lane 1); un-induced for 4 hour (lane 4) and induced with IPTG for 4 hours (lane 5) are shown. Lanes 2 and 3 represent protein profile from cells carrying empty vector and induced with IPTG for 4 hours or un-induced for 4 hours, respectively. Protein molecular weight markers (M) are indicated on the left side. (B).

The expressed recombinant protein is recognized by anti-His antibody. Lane 1: empty vector; and lane 2: induced sample. (C). The anti-PfalMre11 antibody generated against the recombinant PfalMre11 protein (377 amino-acids at the C-terminal region) recognizes the recombinant protein from bacterial lysates. Lane 1: empty vector; and lane 2: induced sample. (D). The anti-PfalMre11 antibody detects a parasite protein of molecular weight 145 kDa (indicated by an arrow). PI: pre-immune sera; I: immune sera.
(TIF)

S4 Fig. Chimera 1 ^{D398N} mutant exhibits much stronger MMS sensitive phenotype than the corresponding yeast mutant ScMre11^{D56N}. Spotting assays on YPD media without (untreated) or with (treated) 0.005% or 0.01% MMS supplementation are shown. The relevant genotypes are shown on the left.
(TIF)

S1 Table. Primers used in this study.
(DOC)

S2 Table. Yeast strains used in this study.
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MKB. Performed the experiments: SBB SAN PV SL SB. Analyzed the data: MKB SB. Contributed reagents/materials/analysis tools: MKB SB. Wrote the paper: MKB.

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