# Role of *Plasmodium falciparum* Proliferating cell nuclear antigens (PfPCNA1 & PfPCNA2) in DNA replication and DNA damage response

***Thesis submitted to Jawaharlal Nehru University for the award of the degree of***

**DOCTOR OF PHILOSOPHY**

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**2016**

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# CERTIFICATE

The research work embodied in this thesis entitled **"Role of *Plasmodium falciparum* Proliferating cell nuclear antigens (PfPCNA1 & PfPCNA2) in DNA replication and DNA damage response"** has been carried out by Ms. Khadija Banu under my guidance at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India. The work presented here is original and has not been submitted in part or full for any degree or diploma of any university/Institution elsewhere.

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DEDICATED TO MY

PRECIOUS

FAMILY

# Acknowledgements

"In the name of Allah, the Most Gracious and the Most Merciful"

First and foremost, my thanks to almighty Allah for the strengths and blessing in completing this thesis.

This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including my well wishers, my friends, colleagues and this institution. Gratitude one feels from heart cannot be discussed exactly.

I take this opportunity to owe immense thanks to my supervisor Prof. Suman Kumar Dhar allowing me to familiarize myself to this fascinating area of Molecular Parasitology. I am greatly indebted to Prof. Dhar for providing necessary infrastructure and resources to accomplish this research work with his enormous support. This work would not have been possible without his guidance and encouragement. It has been a great experience and I am very fortunate to have been guided by him. I would also like to acknowledge Dr. Neelima Mondal for her help in experiments.

I am thankful to Prof. Chinmay Mukhopadhyay for his concern and continued support and informal chatting sessions. I shall always remember his kindness. I would also like to thank Prof. G. Mukhopadhyay, Prof. Rakesh Tyagi, Prof Vibha Tandon, Prof Govardhan Das, Dr. Dipankar Ghosh, Dr. Souvik Bhatacharjee, Dr. Anand Ranganathan and Dr. Saima Aijaz for their support and cooperation over these five years. I am inspired by their excellence in science which encouraged me to aim higher in life.

A special word of thanks goes to my wonderful labmates for creating a cheerful environment and lending a helpful hand whenever needed. They are all more of a friend than colleague. Dr. Vijay, Dr. Srikanta and Dr. Rahul have always been nice to answer basic queries of mine, discussions with them and their suggestions and advices will always be remembered. Mohit has been a good friend and Bhumika, sweet junior were always helpful to me. They both shared neighboring work benches with me in the lab and chit chatting with them during spare time made my stay pleasing during this journey. Krishanu has been very kind and his valuable suggestions are really appreciated. I owe my sincere gratitude for generous and timely help offered by them. My sincere thanks to Meetu, Sabyasachi, Ajay and Priyanka for their kind help at the time of need. Qurat, who joined the lab as a summer trainee too deserves a special thank for helping me in my experiments during final stages of my PhD.

Previous lab members Dr. Atul and Dr. Sandeep with whom I enjoyed my initial days in the lab and their care will always be cherished. Instant questions and technical suggestions were patiently answered by Sandeep sir. I wish to acknowledge their invaluable support and the guidance rendered by them.

I express my heartfelt gratitude to Prof. Devender Sehgal (NII) and Prof. Sandeep Saxena (NII) for letting me to use their facility without which some of the experiments would not have been possible. Hina has been helpful in technical help in my experiments at NII. I would like to thank Pandey ji for his help with animal handling, Gajendra sir (AIRF) for helping me in CD experiments; and Amit & Ganesh Sir for helping me in my confocal experiments.

I would especially like to thank all the technical assistants Amit, Tara Ram ji and Rajesh for performing their jobs with dedication for timely execution of our experiments. I would like to express my sincere thanks to Office staff including Mr. Nand, Ms. Naminita, Ms. Neeti and Mr. Yogender for their cooperation.

All SCMM members and colleagues have made this phase of my life cherishable and memorable.

All my friends including Dr. Seema, Akansha, Priya, Sabir, Rohit, Samreen, Sanju, Pallavi, Sanjay, Amit, Mayank , Mayuri, Jyoti and my all badminton buddies too deserve a special reference who have constantly stood by me and lifted my morale in all the possible ways.

All I can say is that these 5 years of PhD changed the way I looked at science. It has taught me the reductionalist approach, the inquisitive approach and has inculcated into me a habit of targeting big problems at the basics of their root cause. It also taught me that people of science need not always be boring and that they work hard to party harder. So at the end of both this doctoral work and this thesis I feel quiet nostalgic for I am going to miss this protected environment of learning for the rest of my life.

At last but not the least I acknowledge my most sincere gratitude to my dear parents and sisters for their application, inspiration, encouragement, support and blessings during my studies. I am indebted to them for always being there for me. Their blessings and urge have helped me to aim higher in life.

**Khadija Banu**

# ABBREVIATIONS

| | |
|---|---|
| µl | Micro litre |
| Å | Angstrom |
| ACS | ARS Consensus Sequence |
| APS | Ammonium Per Sulfate |
| ARS | Autonomous Replicating Sequence |
| ATP | Adenosine 5'-triphophate |
| BER | Base Excision Repair |
| bp | Base pair |
| BSA | Bovine Serum Albumin |
| CD | Circular Dichroism |
| CDC6 | Cell division cycle protein 6 |
| CDK | Cyclin-dependent protein kinase |
| CDT1 | Cdc10 dependent transcript 1 |
| CQ | Chloroquine |
| DAPI | 4', 6-Diamidino-2-phenylindole |
| DNA | Deoxyribonucleic acid |
| dsDNA | double stranded DNA |
| DTT | Di thiothreitol |
| EDTA | Ethylene Diamine Tetraacetic acid |
| ERCC | Excision repair cross complementation group |
| EXO1 | Exonuclease 1 |
| FenI | Flap endonuclease |
| GAPDH | Glyceraldehyde 3-phosphate Dehydrogenase |
| GFP | Green fluorescent protein |
| GST | Glutathione S-Transferase |

| | |
|---|---|
| Hpi | Hour post infection |
| HU | Hydroxyurea |
| IDC | Intraerythrocytic Developmental Cycle |
| IFA | Immunofluorescence Assay |
| IPTG | Isoproryl-ß-D-thio galactopyranoside |
| Kb | Kilo Base |
| kDa | kilo Dalton |
| M | Marker |
| MBP | Maltose Binding Protein |
| MCM | Mini-Chromosome Maintenance protein |
| MLH1 | MutL homolog 1 |
| MMR | Mismatch repair |
| MMS | Methyl Methane Sulfonate |
| MSH6 | mutS homolog 6 |
| NER | Nucleotide excision repair |
| Ni-NTA | Nickel-Nitriloacetic acid |
| °C | Degree Centigrade |
| ORC | Origin Recognition Complex |
| PAGE | Poly Acrylamide Gel Eletrphoresis |
| PBS | Phospahte Buffer Saline |
| PCNA | Proliferating Cell Nuclear Antigen |
| PCR | Polymerase Chain PCR |
| PIP | PCNA Interacting Protein motif |
| PMSF | Phenyl Methyl Suphonyl Flouride |
| Pol | Polymerase |
| pre-RC | pre-Replication Complex |
| PTM | Posttranslational Modification |

| | |
|---|---|
| qPCR | Quantitative Polymerase Chain Reaction |
| RINGO | Rapid Inducer of G2/M progression in Oocytes |
| rpm | Revolution Per Minute |
| RPMI | Roswell Park Memorial Institute Medium |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| SDS | Sodium-Dodecyl Sulfate |
| Sir | Silent Information Regulator |
| ssDNA | Single Stranded DNA |
| SUMO | Small Ubiquitin-like Modifier |
| TAE | Tris Acetate EDTA |
| TEMED | N,N,N',N',Tetramethylethylenediamine |
| Tris | Tris (hydroxymethyl) amino ethane |
| WB | Western blot |
| WHO | World Health Organization |
| βME | β-Mercapto Ethanol |

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# REVIEW OF LITERATURE

## INTRODUCTION

Infectious diseases are among the biggest challenges for the human existence across the world. Although infectious diseases range from being mild to severe, several of them can be life threatening. One of the most dreadful infectious diseases is known as malaria. Malaria is probably one of the oldest diseases known to mankind and believed to be originated in Africa. Malaria is pre-eminently a tropical parasitic disease and one of the top killers among other communicable diseases including pneumococcal acute respiratory infections, HIV/AIDS and tuberculosis. It is a mosquito borne disease caused by the protozoan parasite *Plasmodium.*

*Plasmodium falciparum* is the highly pathogenic and most deadly parasite causing malaria in humans among other 156 species of *Plasmodium* infecting vertebrates. Out of these, five of them are known to infect humans including *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Caraballo and King 2014). It was discovered in 1880 by Charles Alphonse Laveran, a French Army Surgeon and originally named as *Oscillaria malariae* while examining a drop of blood from a young soldier with fever under a microscope (Mollaret 1980).

Malaria poses a huge social and economic burden on human life. An estimated report of 350-500 million clinical malarial cases are there, most of which are caused by infection with *Plasmodium falciparum* and *Plasmodium vivax*. *P. falciparum* alone causes more than one million deaths each year, 90% of which occur in sub-Saharan Africa, and it is considered to be endemic around the equator. 77% of which occur in children under five in synergy with other infections and illnesses. WHO 2015 report shows a dramatic decline in the global malaria burden since 2000. 57 countries have reduced their malaria cases by 75%. Figure 1 represents a decrease from 106 countries and territories to 95 of ongoing malaria transmission since 2000. According to the October, 2015 WHO estimates, about 214 million new cases of malaria were registered in 2015 with an estimated deaths of 4,38,000 (Table 1). A global decrease by 37% in malaria incidence

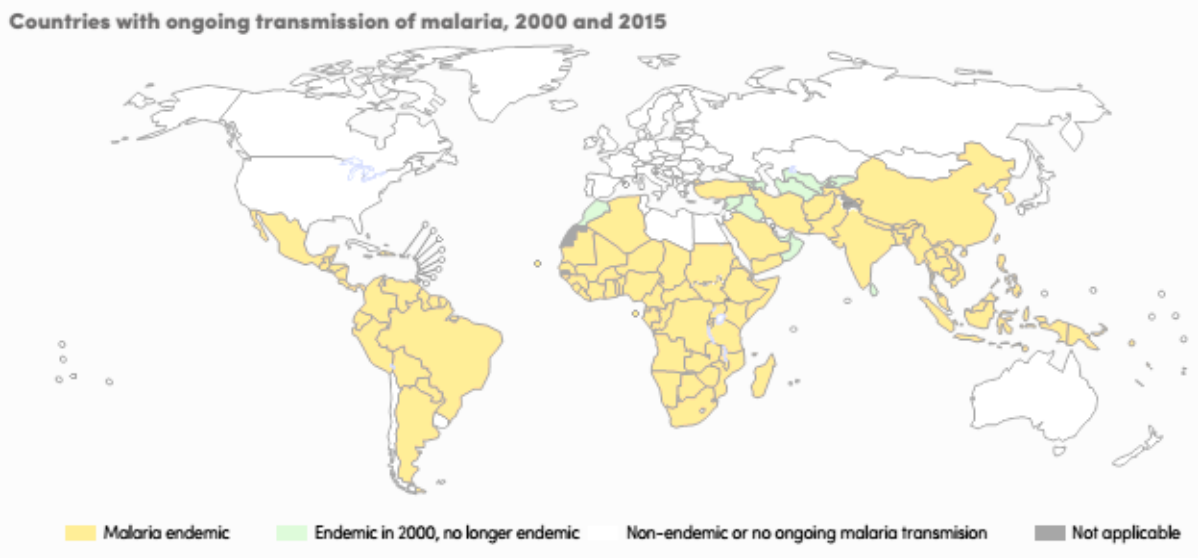


**Figure 1. Global transmission of Malaria since 2000.** The number of countries and territories with ongoing malaria transmission has decreased from 106 countries and territories to 95 since 2000 to 2015. Data included 95 countries and territories and six countries that have recently eliminated malaria (World malaria report, 2015).

and a 60% decrease in global malaria mortality rates have been reported between 2000 and 2015. Currently, India accounts for two-thirds of all confirmed malarial cases of South-East Asian region, with Orissa, Jharkhand, West Bengal, Madhya Pradesh and Chhattisgarh contributing to the bulk (60 %) of the malaria (World Malaria Report, 2015). Despite this tremendous progress, much needs to be done for further reduction in malaria burden.

With an increasing resistance to the commonly used drugs like quinine, chloroquine, sulphadoxine etc. against the malaria parasite, it has become a matter of global concern and there is an urgent necessity to identify new drug targets for therapeutic intervention. Studying different aspects of *P. falciparum* biology to understand various underlying mechanisms of the disease pathogenesis may offer scope and hope to combat such a deadly disease. DNA replication is one such key biological process essential for both parasite survival and multiplication. DNA replication machinery involves an integrated activity of several groups of multi-subunit proteins and offers to be a good strategic multidrug targeted approach.

**Estimated malaria cases and deaths, by WHO region, 2000–2015**

| | Estimated number of malaria cases (000's) | | | | Change | Estimated number of malaria deaths | | | | Change |
|---|---|---|---|---|---|---|---|---|---|---|
| **WHO region** | **2000** | **2005** | **2010** | **2015** | **2000–2015** | **2000** | **2005** | **2010** | **2015** | **2000–2015** |
| African | 214 000 | 217 000 | 209 000 | 188 000 | -12% | 764 000 | 670 000 | 499 000 | 395 000 | -48% |
| Americas | 2 500 | 1 800 | 1 100 | 660 | -74% | 1 600 | 1 200 | 1 100 | 500 | -69% |
| Eastern Mediterranean | 9 100 | 8 600 | 4 000 | 3 900 | -57% | 15 000 | 15 000 | 7 000 | 7 000 | -51% |
| European* | 36 | 5.6 | 0.2 | 0 | -100% | 0 | 0 | 0 | 0 | |
| South-East Asia | 33 000 | 34 000 | 28 000 | 20 000 | -39% | 51 000 | 48 000 | 44 000 | 32 000 | -37% |
| Western Pacific | 3 700 | 2 300 | 1 700 | 1 500 | -59% | 8 100 | 4 200 | 3 500 | 3 200 | -60% |
| **World** | **262 000** | **264 000** | **243 000** | **214 000** | **-18%** | **839 000** | **738 000** | **554 000** | **438 000** | **-48%** |
| Lower bound | 205 000 | 203 000 | 190 000 | 149 000 | | 653 000 | 522 000 | 362 000 | 236 000 | |
| Upper bound | 316 000 | 313 000 | 285 000 | 303 000 | | 1 099 000 | 961 000 | 741 000 | 635 000 | |

* There were no recorded deaths among indigenous cases in the WHO European Region for the years shown.
***Source:*** *WHO estimates*

**Table 1. Estimated case and deaths due to malaria according to WHO report, 2015.** A large reduction is seen in the number of malaria cases and deaths since 2000. The number of malaria cases fell from an estimated 262 million globally in 2000, to 214 million in 2015, thereby reporting a decline of 18%. The number of deaths due to malaria fell from an estimated 8, 39, 000 in 2000 to 4, 38, 000 in 2015, a decline of 48% globally. Most cases and deaths in 2015 are estimated to have occurred in the WHO African Region (88%), followed by the WHO South-East Asia Region.

## Life cycle of *Plasmodium falciparum* - the malarial parasite

*P. falciparum* is the most virulent and hence the most studied form of human pathogen. It completes its life cycle within two different hosts; the female Anopheline mosquito and the human. Within the human host, parasite invades both the hepatocytes as well as erythrocytes, but it is the erythrocytic phase of its life cycle (48 hours) that causes severe pathogenesis of malaria. The first symptoms of malaria include fever, headache, chills and vomiting usually appearing between 10 to 15 days after the mosquito bite. Without prompt treatment, *P. falciparum* malaria can lead to severe illness and death.

*P. falciparum* has a complicated life cycle and involves an Exo-erythrocytic, Erythrocytic and a Sporogonic cycle. It starts with the bite of an infected female Anopheles mosquito during a blood meal from a human host. *P. falciparum* sporozoites enters the human body and invade the human liver cells (hepatocytes) where they undergo multiple

rounds of replication (Exo-erythrocytic cycle) and differentiate to form merozoites. These merozoites upon release invade the host's red blood cells (RBCs). Inside the erythrocytes, the parasite in its early stages adopts a ring like morphology and progresses towards the trophozoite stage where it replicates its DNA, subsequently giving rise to multinucleated schizont containing 16-32 merozoites. Another cycle of asexual develop-

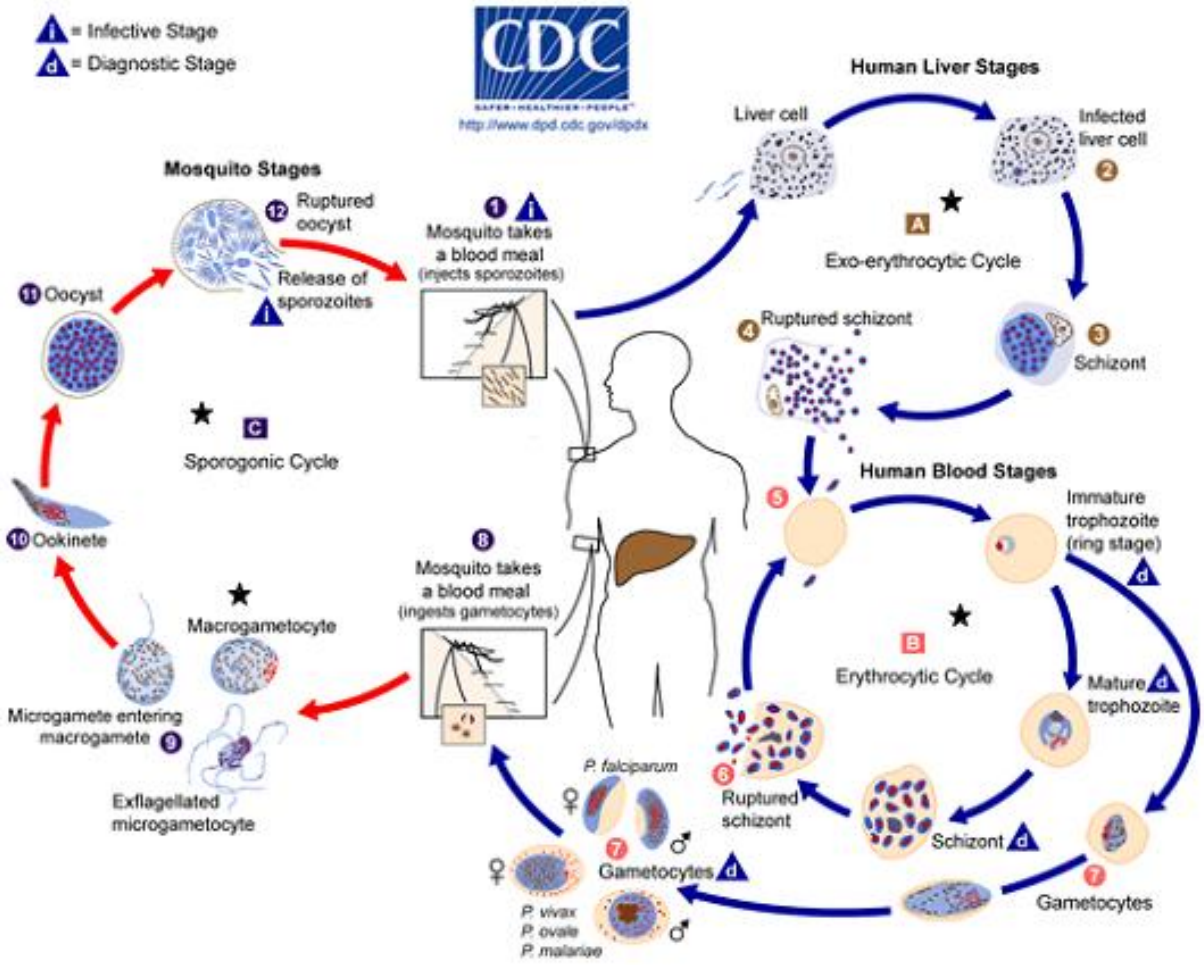


**Figure 2.** ***Plasmodium falciparum*** **life cycle in female anopheline mosquito and human host,** depicting various stages and approximate duration. Various stages where DNA replication may take place are indicated as (★) (http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm.).

-ment starts with the release of merozoites when a schizont ruptures to infect fresh erythrocytes (Bannister, Hopkins et al. 2000, Garcia, de Azevedo et al. 2008, Antinori, Galimberti et al. 2012). Some of these merozoites during schizogony differentiate into

sexual forms - female (macrogametocytes) and male (microgametocytes), respectively, which when ingested by a female anopheline mosquito leads to sporogonic (sexual) cycle. Once in its midgut, the macrogametocyte is released by the erythrocytes to become a macrogamete whereas the microgametocyte divides its nucleus to form 8 flagellated microgametes. On fertilization, the zygote is formed which results into a slowly motile form, termed ookinete. 24-48 hours after the ingestion of blood, the oocyst develops. Subsequently, repeated nuclear division results in the formation of a mature oocyst containing thousands of sporozoites. These sporozoites reach the salivary glands of the insect and become highly infective within 24 hours and are ejected during skin probing by the insect.

Organization of cell division in *Plasmodium* is unique from model organisms because multiple rounds of DNA replication takes place at five distinct points of its life cycle, two of which takes place in the human host, i.e. in the hepatocytes (exo-erythrocytic schizogony) and in the erythrocytes (during erythrocytic schizogony). Thehe remainder occurs in the mosquito vector during gametogenesis; following fertilization, just before meiosis takes place; and in oocysts during the formation of sporozoites (White and Kilbey 1996) (Figure 2). Moreover, parasite's genome is highly A-T rich (~80%). It is worth mentioning here that the massive genome of 23 megabase is replicated thrice (majorly) during the life cycle of the malaria parasite within a relatively short span of time making them more prone to DNA damage. To combat this, the parasite must have developed very efficient replication machinery as well as a reliable repair mechanism. Thus, it will be quite interesting to know the mechanism of parasite replication which causes a huge turnover in its number following DNA replication and cell division.

**DNA REPLICATION**

DNA replication is a fundamental and indispensable cellular process that precedes cell division. The general features of DNA replication are conserved across various eukaryotes and require a specific set of proteins for the nucleic acid metabolism pathway. Failure to achieve an accurate and faithful duplication of the genome will result in catastrophic genomic instability. Here, we present an overview of the

chromosomal replication in intraerythrocytic developmental cycle (IDC) of *P. falciparum* with respect to the well studied eukaryotic model systems including mammalian cells and yeast.

The process of DNA replication starts in a coordinated manner at DNA elements termed as **origins of replication** (Lundgren, Andersson et al. 2004, Robinson, Dionne et al. 2004). In *Saccharomyces cerevisiae* these origins are short sequence-specific DNA elements, known as autonomously replicating sequences (ARS) whereas in metazoans these origins of replication are much less defined at the sequence level. However, by now no such conserved origin sequences have been reported in *P. falciparum* but the presence of a high AT content in the parasite genome raises the possibility of the propensity of AT rich sequences to act as initiator sequences. Also, a high rate of genome duplication may lead us to speculate that there may not be sequence specificity like origins in early embryos of *Xenopus laevis* and *Drosophila melanogaster*.

During initiation step of DNA replication, initiator proteins recognize the origin, followed by a sequential recruitment of additional proteins facilitating DNA unwinding as a preparatory phase of DNA replication. It requires the formation of a **pre-Replicative Complex** (pre-RC) which paves the way for the replication fork progression or elongation (Dutta and Bell 1997). It involves origin licensing and the complex contains a six subunit ATPase called the **origin recognition complex** (ORC; Orc1-6) (Bell and Dutta 2002, Diffley 2004). In *P. falciparum*, ORC1-5 subunits have been identified based on homology and experimental evidences (Deshmukh, Srivastava et al. , Li and Cox 2003, Mehra, Biswas et al. 2005, Gupta, Mehra et al. 2008) ORC binding to origin is then followed by binding of ATPase **Cdc6** and licensing factor **Cdt1**. Now, the hexameric complex of helicases called **MCM2-7** is recruited at this ORC/Chromatin site which is now licensed for initiation (Labib, Tercero et al. 2000). *Plasmodium* genome like other eukaryotes, encodes six MCM subunits with signature MCM motif and a conserved WALKER A type ATP binding domain (Young and Tye 1997, Patterson, Robert et al. 2006).

The next step involves the formation of a replication fork at the origin which is promoted by subsequent recruitment of additional factors (Mcm10, Cdc45, Dpb11, Sld2, Sld3, and the GINS complex in *S. cerevisiae*) and activation of S-phase cyclin-dependent kinases (CDKs) and the Cdc7-Dbf4 kinase (DDK), that phosphorylate proteins of the replisome (e.g., Mcm proteins, Sld2, Sld3) and other targets. This in turn leads to the assembly of the replicative helicase together with associated factors for further recruitment of the DNA polymerases and other factors required for DNA synthesis (Moldovan, Pfander et al. 2007). The DNA duplex unwound by the action of helicases and the resulting single-stranded DNA is stabilized through binding of multiple heterotrimeric single-strand binding protein **RPA**, and a bidirectional **replication fork** is formed. The largest subunit *Plasmodium falciparum* replication protein A (PfRPA1) has only been isolated and identified in the parasite extract and protein expression profile of PfRPA1 in intraerythrocytic asexual stages coincides with the replication phase in late trophozoites and schizonts, which parallels the expression profile of other DNA replication proteins (Voss, Mini et al. 2002).

During elongation step of DNA replication, the two strands of DNA are synthesized by different mechanisms. The leading strand replicates in a continuous fashion through the 5′-to-3′-polymerase activity of **DNA polymerases**, while lagging strand synthesis occurs discontinuously, in the form of short fragments termed as Okazaki fragments. In *P. falciparum,* DNA polymerase α and DNA polymerase γ have been purified and characterized from crude extract (Pol α and γ) and mitochondrial DNA polymerases has also been reported (Pol γ) (Chavalitshewinkoon, de Vries et al. 1993, Chavalitshewinkoon-Petmitr, Chawprom et al. 2000). Pol δ has also been found to be conserved in *P. falciparum* and it shows a possible involvement in Base Excision Repair pathway (Hubscher, Nasheuer et al. 2000). Also, a DNA polymerase beta like enzyme has been identified and partially purified from large scale *P. falciparum* parasite culture (Nunthawarasilp, Petmitr et al. 2007).

For synthesis of the complementary DNA strand, initially a short RNA primer is made by the **primase** enzyme, followed by a short stretch of DNA synthesized by **polymerase**

**α** (Polα). Small subunit of **Pfprimase** possess the features of catalytic center of DNA polymerase, reverse transcriptase and RNA polymerase and the purified protein has demonstrated its ability to initiate *de novo* primer formation (Prasartkaew, Zijlstra et al. 1996).

**Replication factor C** (RFC) then binds at the primer template junction to catalyze the loading of the trimeric processivity factor **PCNA** (Pol30 in *S. cerevisiae*) which encircles DNA due to its ring like structure leading to an association with the replicative polymerases, Polδ or Polε; which take over from Polα to carry out the processive DNA synthesis. *In vivo* expression profiling reveals that **PfRFC5** peaks in late trophozoites / early schizont stages corresponding to DNA replication phase of the parasite (Le Roch, Zhou et al. 2003). PfRFC5 has also been identified to interact with PfMCM6 and a CDK, PfMRK in a bacterial two-hybrid screen. PfMRK was found to phosphorylate both PfRFC-5 and PfMCM6 by *in vitro* assays (Jirage, Chen et al.).

*P. falciparum* has been reported to have two PCNAs named PfPCNA1 and PfPCNA2 (Kilbey, Fraser et al. 1993, Li, Warren et al. 2002, Patterson, Whittle et al. 2002), and the present study will focus on the role of these molecules in nucleic acid metabolic pathways of the malarial parasite. A detailed overview of PfPCNAs will be discussed later.

During lagging strand synthesis, the replicative polymerase partially displaces the encountered Okazaki fragment and a flap structure is generated which is excised by the activity of **flap structure-specific endonuclease-1** (FEN-1, Rad27 in *S. cerevisiae*), and the resulting nick is sealed by **DNA ligase I** (Cdc9 in *S. cerevisiae*). PfFenI was reported to have DNA structure-specific flap endonuclease and 5'-3' exonuclease enzymatic activities similar to other species (Casta, Buguliskis et al. 2008). It carries an extended C-terminus, a more internally located PCNA-binding site while the N-terminus domain and intermediate domain show high degree of conservation (Warbrick 1998, Gary, Park et al. 1999, Sakurai, Kitano et al. 2005). Buguliskis, *et al,* identified and characterized PfLigI. It has been shown in an *in vitro* DNA repair synthesis assay, that PfFEN-1

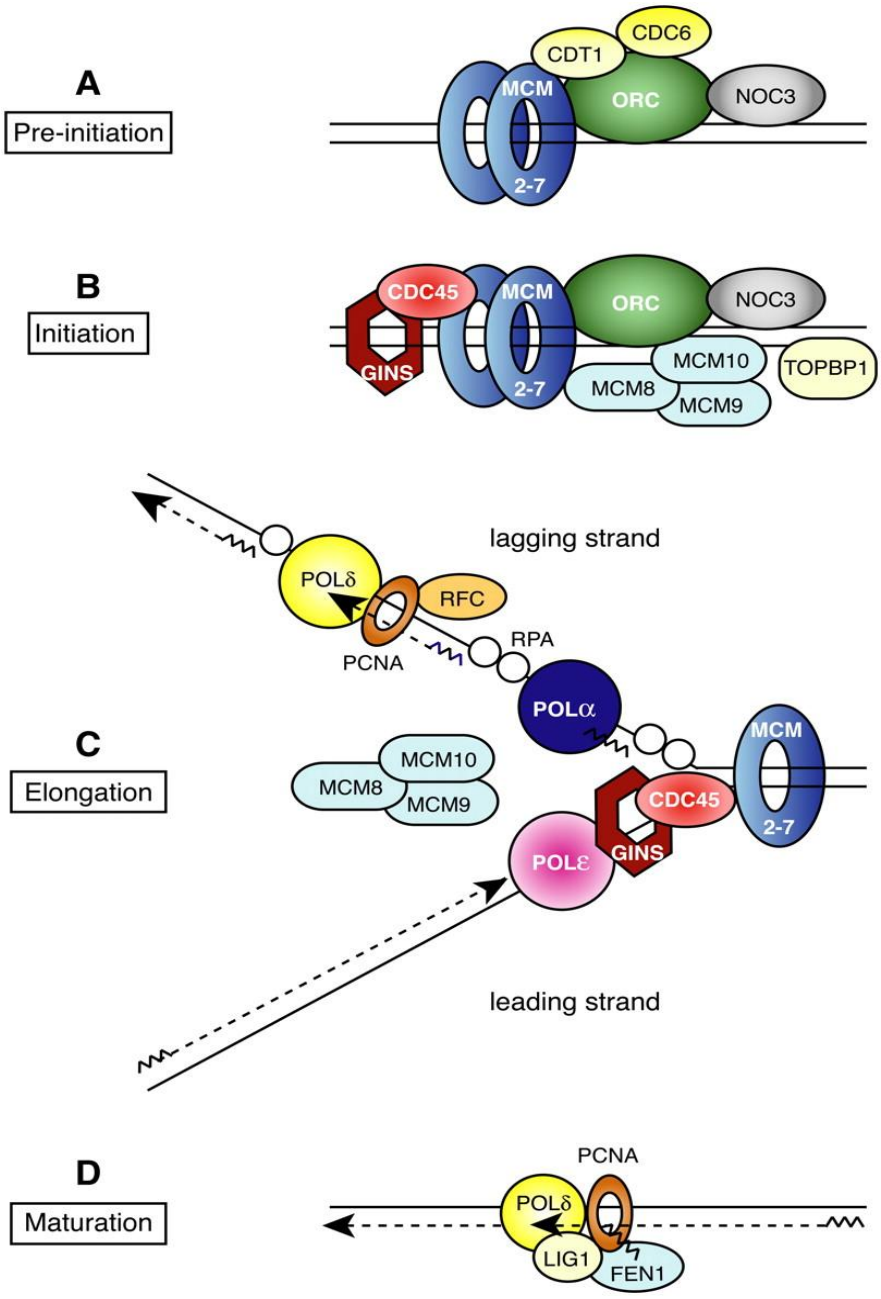


**Figure 3. The basic mechanism of DNA replication showing various components of a Pre-RC complex and a replisome. (A)** The formation of a pre-replication complex occurs with the sequential binding of replication factors (like ORC 1-6, Cdc6, MCM 2-7, etc.) to the origin. **(B)** Initiation involves the formation of a replication fork promoted by binding of additional factors like GINS, Cdc-45, etc. at the origin. **(C)** Helicases and RPA assist in elongation step carried out by DNA polymerases triggering DNA replication to occur in a bidirectional way. The leading strand is synthesized continuously while the lagging strand in discontinuous short patches. **(D)** Maturation involves removal of the flap structures of lagging strand by FEN1 and sealing of the nick by DNA Lig1.

generated nicked DNA substrate that can be ligated by recombinant PfLigI (Buguliskis, Casta et al. 2007). Based on the above discussion, Figure 3 is shown to illustrate the basic mechanism of DNA replication in eukaryotes.

The present study mainly focuses on Processivity factor; therefore we will try to review them in greater detail as it performs a plethora of functions within a cell due to its versatility.

**1. PCNA- Proliferating cell nuclear antigen – processivity factor**

Proliferating cell nuclear antigen (PCNA) is a protein central to DNA replication machinery as it acts as scaffold to recruit many factors essential during DNA synthesis thus acting as a molecular coordinator of the core DNA replication machinery.

Primarily, it provides processivity to the replicating polymerases ε and δ (Waga and Stillman 1998, O'Donnell, Langston et al. 2013) by forming a homotrimeric ring around the DNA strands (prokaryotic homologue of β clamp / product of gene-45 of bacteriophage T4).

PCNA was discovered as an antigen recognized by certain autoantibodies from patients suffering from an autoimmune disease Systemic lupus erythmatoses (Miyachi, Fritzler et al. 1978). Later, in 1982 another study identified a nuclear protein termed as Cyclin since its expression is regulated such that the quiescent cells show very low levels and increases several fold after serum stimulation during S phase of the cell cycle. (Bravo, Fey et al. 1982). Further, it was demonstrated to be the auxillary factor for DNA polymerase. Proliferating cell nuclear antigen (PCNA) and cyclin have common properties and they are identical to each other (Bravo, Frank et al. 1987, Prelich, Tan et al. 1987).

**1.1 Structure**

DNA sliding clamps are structurally and functionally conserved across all the branches of life. Whether it is homodimeric β clamp of eubacteria, a homotrimeric ring in eukaryotes, T4 phage; or a heterotrimer as in case of archaea they all have a

superimposable three dimensional structures, despite of any sequence similarity (Krishna, Kong et al. 1994, Gulbis, Kelman et al. 1996). They all form a ring with a pseudohexameric symmetry encircling the DNA to be able to slide freely in either direction. Each eukaryotic PCNA monomer has two globular domains which oligomerizes in a head to tail arrangement to form a trimeric ring (Kong, Onrust et al. 1992). The inner face of the ring is formed of alpha helices and bears a net positive charge due to the presence of several lysine and arginine residues which promotes Clamp DNA association; and beta sheets are present on the outer surface of the trimer where a strong negative electrostatic potential exist which may prevent nonspecific interaction with DNA. (Krishna, Kong et al. 1994, Gulbis, Kelman et al. 1996).

It has been demonstrated that residue Y114 of PCNA is important for the monomers to keep their overall structure for trimerisation which is essential for interaction with other proteins like RF-C for loading of DNA polymerase delta (polδ) onto DNA (Jonsson, Podust et al. 1995). Another study also showed the importance of β sheets in ring stability since a single amino acid substitution in the β sheet lining the interface (where Ser was replaced by Pro) resulted in the monomerization of the protein (Ayyagari, Impellizzeri et al. 1995).

**1.2 PCNA and its interactions**

PCNA has been referred to as a "sliding clamp" since it acts as a moving platform for factors involved in the process of DNA replication/repair by mediating interactions in a sequence-independent manner. PCNA trimeric ring has a distinct front and back sides. The front side where the globular domains with N- and C- terminus are linked through an interdomain connecting loop (IDCL) and the back side has loops to connect β strands protruding outwards (Gulbis, Kelman et al. 1996).

PCNA trimer acts as a loading dock thereby coordinating various DNA replication and repair functions by allowing simultaneous binding of DNA polymerases and other proteins (complexes). Each subunit of the PCNA trimer possesses one such binding site. Three molecules of human FEN-1 have also been co-crystallized with a single PCNA

molecule, supporting the concept that multiple proteins can be simultaneously accommodated on one PCNA ring (Sakurai, Kitano et al. 2005).

PCNA interacts with its protein partners through its **Center loop** (41DSSH44) and the **C-terminal tail** located beside the Center loop on the **C- or front side** (254KIE257), and the **interdomain-connecting loop** (121LDVEQLGPEQE132) on the outer surface. It has been suggested that the front side of the PCNA ring faces the 3' end of the replication primer, while the back side faces replicated DNA (Fukuda, Morioka et al. 1995, Jonsson, Hindges et al. 1998). Certain proteins may bind to the back side to coordinate DNA replication with various other functions coupled to DNA replication or repair process (Fukuda, Morioka et al. 1995). All known PCNA-associating proteins

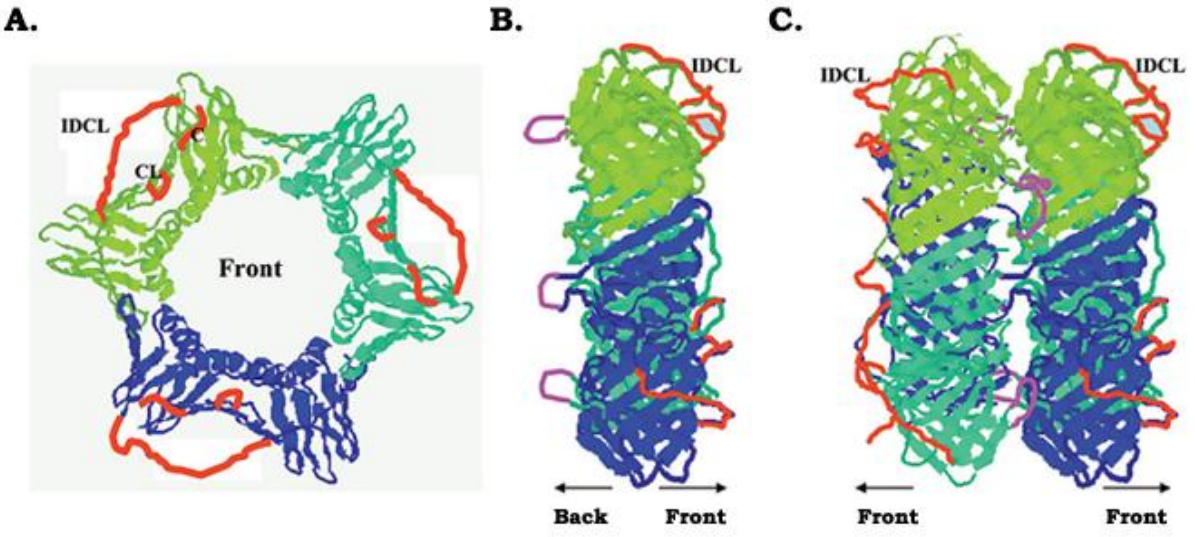


**Figure 4.** A three-dimensional PCNA trimer shown from front **(A)** and side view **(B)**. Interdomain connecting loop, IDCL; center loop, CL and C-terminus, C are indicated and shown in red. **(C)** A PCNA double trimer model showing back -to-back interaction of PCNA monomers (Naryzhny 2008).

bind to the front side or outer surface, except pol ε which has been shown to bind both the front and back side by Maga et al. (Maga, Jonsson et al. 1999). Many cellular functions coupled with DNA replication such as chromatin remodeling, DNA methylation and mismatch repair processes may take place through the formation of a PCNA **double trimer**. Such a model has been proposed and Arg5 and Lys 110 residues are found to be critical for a back -to-back interaction of PCNA monomers for a double

trimer formation and PCNA double trimer can simultaneously accommodate both DNA polymerase δ and CAF-1 complexes, this may not be possible by a single homotrimer (Naryzhny, Zhao et al. 2005).

The ability of PCNA to interact with multiple protein partners having disparate structures is explicable at least in part through the existence of a PCNA-binding motif that is present on a number of its binding proteins (Warbrick 1998). This occurs through a conserved PCNA binding domain termed the **PCNA interacting protein (PIP)** motif acting as a predictor of PCNA-binding capacity (Warbrick, Heatherington et al. 1998).

An alignment of these binding motifs shows that it consists of a consensus sequence Q-xx-(h)-x-x-(a)-(a) (where "h" represents residues with moderately hydrophobic side chains, e.g., L, I, M; "a" represents residues with highly hydrophobic, aromatic side chains, e.g., F, Y; and "x" is any residue). p21, Fen1, RFC, XPG, Dacapo protein, which is a cyclin-dependent kinase inhibitor, the Pogo transposon are some examples of proteins bearing PIP motif.

Because of its ability to interact with a wide array of proteins, multifunctional nature of PCNA protein is facilitated as elucidated by its involvement in other aspects of nucleic acid metabolism including DNA repair, translesion DNA synthesis, DNA methylation, chromatin remodeling, cell cycle regulation, sister chromatin cohesion, apoptosis, gene expression (Kelman 1997, Maga and Hubscher 2003).

Later, a second, larger group of peptides containing a different consensus sequence, K-A-(A/L/I)-(A/L/Q)-x-x-(L/V) for PCNA binding was discovered and termed as **KA box**. (Xu, Zhang et al. 2001). Database search and analysis show that many proteins contain the second consensus sequence. These include proteins that are involved in DNA replication (DNA pol δ and pol ε, MCM members), DNA repair [three XP proteins (XPC, XPG, XPD), mismatch repair protein MSH6, cyclin D3 and BRCA1 and BRCA2)]. However, nuclear uracil-DNA glycosylase (UNG2), a protein involved in Base excision repair (BER) contains a PIP-box at the N-terminus and also a PCNA-binding KA motif (Xu, Zhang et al. 2001).

Not all known PCNA-binding proteins contain the PIP box sequence, and the existence of a second motif may provide an explanation and may be of utility in identifying PCNA-binding domains in candidate proteins that bind to PCNA.

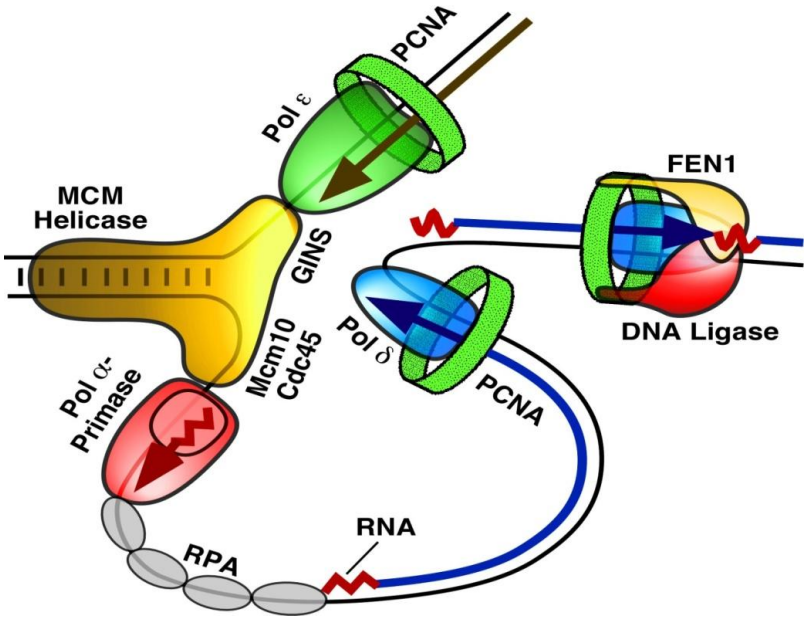


**Figure 5. PCNA with its interactors in a DNA replication pathway.** During DNA replication PCNA interacts with DNA pol ε and δ on leading and lagging strands, respectively. It also interacts with FEN1 and DNA ligase. (Carol A. Rouzer, VICB Communications, August 27, 2010).

### 1.3 PCNA in DNA replication

PCNA's role in DNA replication is quite evident from the fact that it plays an essential role of the auxiliary factor for the processive DNA polymerases. Dramatic changes in PCNA's nuclear distribution during S phase of the cell cycle. Early in S phase, it is localized throughout the nucleoplasm except nucleoli with gradual appearance of punctated pattern with foci throughout the nucleus, including nucleolus as well during late S phase and such a distribution of this protein is not a consequence of transformation since it is observed in normal cells as well (Celis and Celis 1985). This suggested PCNA to play a role in DNA replication or to be a part of replication machinery. However, a direct evidence came through the identification of PCNA as a

component required for the in vitro replication of SV-40 (Prelich, Tan et al. 1987). Other *in vitro* and *in vivo* studies demonstrated PCNA's role in coordinating leading and lagging strand synthesis. Using fractionated cell extract, in an SV-40 *in vitro* replication assay, it was shown that leading strand DNA synthesis was abolished in the absence of PCNA and lagging strand DNA replication was abnormal.(Prelich and Stillman 1988).

Downey et al., 1990 have shown a dramatic increase in processivity of Pol δ in the presence of PCNA and RFC. The amino acid sequence analysis along with biochemical and immunological studies confirmed that the auxillary protein of Pol delta and PCNA were identical and established the role of PCNA in DNA replication. PCNA also serves as the sliding clamp for DNA polymerases ε (Yuzhakov, Kelman et al. 1999, Maga, Stucki et al. 2000, O'Donnell, Langston et al. 2013) and interacts with other proteins involved in DNA replication. It recruits FEN-1 endonuclease, for the maturation of an Okazaki fragment by removing the RNA part (Waga and Stillman 1998). Later, the gap is filled and Cdc9 DNA ligase ligate the two adjacent fragments (Levin, McKenna et al. 2000). Therefore, PCNA not only plays a role in DNA synthesis as part of the polymerase holoenzyme but also in the final steps of DNA replication (Figure 5).

### 1.4 PCNA in DNA Damage Response

Proliferating cell nuclear antigen (PCNA) is essential for DNA replication machinery (Majka and Burgers, 2004) and DNA repair (Moldovan et al., 2007). Early Immuno-histochemical studies showed nucleus stained strongly with anti-PCNA antibodies following DNA damage by UV-irradiation although the cells were not in S-phase. This observation suggested a role for PCNA in DNA repair (Celis and Celis 1985, Toschi and Bravo 1988). Prosperi *et al.*, have shown following UV irradiation human quiescent fibroblasts shows three-fold increase in PCNA levels relative to that of unirradiated control cells. Moreover, it has been shown that DNA damage by UV induces chromatin enrichment of mammalian PCNA (Prosperi, Stivala et al. 1993).

In last few decades, the involvement of PCNA in DNA excision and mismatch repair processes has become quite evident (Nichols and Sancar 1992, Shivji, Kenny et al. 1992,

Johnson, Kovvali et al. 1996, Umar, Buermeyer et al. 1996) through *in vitro* repair assays as well as in repair of double strand breaks (DSBs) (Holmes and Haber 1999, Dorazi, Parker et al. 2006). Furthermore, *in vivo* studies conducted on the *Drosophila melanogaster mus209* mutant demonstrated an important role for PCNA in DNA repair (Henderson et al., 1994).

Nucleotide excision repair (NER) (Sancar, Lindsey-Boltz et al. 2004) removes bulky DNA lesions generated by exposure to radiation or chemicals. XPC protein (Rad4 in S. *cerevisiae*) first detects the lesion. Subsequently, XPD (ERCC2 in human and Rad3 in *S. cerevisiae*) and the XPB (ERCC3) helicases of the TFIIH transcription DNA-repair factor complex open up the DNA around the lesion, and the endonucleases XPG (ERCC5; Rad2 in *S. cerevisiae*) and XPF (ERCC1) cut the strand near damage. The single-stranded gap is finally closed by DNA synthesis and ligation. Gary *et al*. demonstrated PCNA's role in NER pathway as XPG carries a PIP motif at its C-terminus for PCNA binding. It seems likely that PCNA coordinates DNA excision and synthesis (Nichols, 1992, Shivji 1995, Aboussekhra 1995).

Base excision repair (BER) (Sancar, Lindsey-Boltz et al. 2004) repairs chemical alterations of the nucleotide bases in DNA, including alkylated, oxidized, reduced, or deaminated bases, as well as misincorporated uracils. DNA glycosylase recognize modified bases and removes the bases from the nucleotides, forming abasic sites. The apurinic/apyrimidinic (AP) endonuclease cleaves the phosphodiester bond 5′ of the abasic site. BER follows in two different ways. In Short-patch repair, Polβ fills the single nucleotide gap, and the nick is sealed by the XRCC1-DNA ligase 3 complex and in Long-patch repair, the RFC-PCNA-Polδ/Polε complex is recruited to perform repair DNA synthesis, analogous to lagging strand synthesis involving FEN-1 and DNA ligase I (and sometimes the PCNA-related 9-1-1 complex) Matsumoto, 1994; Matsumoto, 1999; Fortini, 1998; Gary, 1997)

Mismatch repair (MMR) (Jiricny 2006) another repair pathway corrects base-base mismatches and small insertion/deletion loops. In this, the error-containing portion of the synthesizing DNA strand is removed followed by targeting of DNA synthesis

machinery to the newly formed single-stranded gap. Components of MMR pathway like MSH6, MSH3, MLH1, and EXO1, interact directly with PCNA and at least MSH6, MSH3, and MLH1 possess PIP boxes. A stepwise recruitment of these factors to PCNA allows MMR to function in an ordered pathway (Umar, 1996).

Other than the DNA repair mechanisms, cells have evolved bypass mechanisms to handle stalled replication forks. One important way is to switch from accurate replicative polymerases (i.e., Pol δ or Pol ε) to the error-prone translesion polymerases (TLS) that can read through lesions as they can accommodate even bulky lesions at their active sites. More accurate or error free bypass mechanism operates through switching of templates to use the undamaged information of the sister duplex (Barbour and Xiao 2003). Bypass modes depend on covalent modifications of ubiquitin to PCNA. Monoubiquitylation of PCNA at lysine 164 catalyzed by Rad6 (E2) and Rad18 (E3) triggers the error-prone pathway through TLS, whereas PCNA polyubiquitylation gives way to error-free mode of the bypass (Kannouche and Lehmann 2004, Kannouche, Wing et al. 2004, Watanabe, Tateishi et al. 2004). Monoubiquitylated PCNA has been demonstrated to interact with the TLS polymerases Polη (Rad30) and Polζ (Rev3/Rev7) in yeast (Stelter and Ulrich 2003) as well as humans. Also, SUMOylation of PCNA K164 and K127 by Siz1 E3 SUMO ligase helps prevent uncontrolled recombination (Papouli, Chen et al. 2005, Pfander, Moldovan et al. 2005).

Chromatin assembly factor 1 (CAF-1), a molecular chaperone that deposits histones H3 and H4 onto newly replicated DNA requires its direct interaction with PCNA via a PIP box of its largest subunit Cac1, to efficiently carry out its function (Zhang, Shibahara et al. 2000, Krawitz, Kama et al. 2002). In yeast PCNA mutants defective in this interaction does not allow CAF-1 to be properly loaded on chromatin. These cells exhibit gene-silencing defects. PCNA-dependent recruitment of CAF-1 was also observed at sites of NER, where it might re-establish chromatin after the repair process is finished (Gaillard, Martini et al. 1996). CAF-1-dependent chromatin assembly appears crucial for replication bypass by the RAD6 pathway as well (Game and Kaufman 1999), indicating that PCNA-CAF-1-dependent chromatin assembly is perhaps generally

important for events that require DNA synthesis. Studies also indicate that binding of PCNA to the transcriptional co-activator p300 is important for chromatin remodeling at DNA lesions to facilitate PCNA function in DNA repair (Hasan, Hassa et al. 2001).

However, PCNA plays a role in events post replication such as cytosine methylation and chromatin assembly (Chuang, Ian et al. 1997, Moggs, Grandi et al. 2000); chromatin remodeling, sister chromatin cohesion and other cellular functions including cell cycle regulation, apoptosis, gene expression (Kelman 1997, Maga and Hubscher 2003) which is beyond the scope of this study, so we have not covered the overview of those PCNA functions. However, this does not underestimate its role in these pathways within a cell.

### 1.5 PCNA Regulation through post-translational modifications

The diverse function of proliferating cell nuclear antigen (PCNA) is thought to be due, in large part, to posttranslational modifications. How does a single protein coordinate two tightly linked processes i.e. DNA replication and DNA repair that may require a large number of interacting proteins; still remains an open question? It is possible through regulation by means of reversible post-translational modifications. PCNA has been widely studied and reported to undergo PTMs like Ubiquitination, Sumoylation, Phosphorylation, Acetylation and Methylation.

As discussed above in the event of a stalled replication fork, ubiquitination of PCNA occurs for a polymerase switch. Another modification of PCNA at K164 by the ubiquitin-like protein SUMO in budding yeast *S. cerevisiae* has been reported during S phase by the SUMO-specific E2 Ubc9 and the SUMO ligase Siz1, which occurs in a damage-independent manner (Hoege, Pfander et al. 2002). Sumoylation of PCNA has also been shown to prevent the resolution of damage site by homologous recombination to promote ubiquitin-dependent lesion bypass (Papouli, Chen et al. 2005, Pfander, Moldovan et al. 2005). Recruitment of Srs2 (an anti- recombinogenic enzyme) prevents this recombination to occur in replicating cells. Lack of PCNA sumoylation during mitosis leads to an increased spontaneous sister chromatid recombination and causes infidelity in chromosomal transmission to daughter cells (Robert, Dervins et al. 2006).

PCNA acetylation and deacetylation also play an important role in DNA replication by its binding to DNA polymerases. Three distinct PCNA isoforms exist that include moderately acetylated main (M) form in all of the subcellular compartments. The highly acetylated acidic form is found in the nucleoplasm, nuclear matrix, and chromatin while a deacetylated basic form is found in the nucleoplasm of cycling cells. Deacetylation of PCNA leads to reduced interaction with DNA polymerases β and δ. Co-immunoprecipitation of p300 and histone deacetylase (HDAC1) with PCNA is likely to be involved in acetylation and deacetylation, respectively of PCNA. It has been suggested that the acetylated forms participate in DNA replication, while the deacetylated form is associated with the termination of DNA replication since deacetylated PCNA complexes show lower DNA polymerization activities than highly acetylated PCNA complexes (Naryzhny and Lee 2004).

Apart from ubiquitination and sumoylation, there are evidences suggesting PCNA to undergo phosphorylation as well. Wang, et al. demonstrated that activated nuclear epidermal growth factor receptor (EGFR) phosphorylates PCNA in the nucleus by its tyrosine kinase activity. Phosphorylated PCNA is stably associated onto the chromatin and blocking phosphorylation leads to destabilization of PCNA bound form by an unidentified mechanism. Y211 phosphorylation of PCNA showed pronounced cellular proliferation, and is correlated with poor survival of breast cancer patients, as well as nuclear EGFR in tumors (Wang, Nakajima et al. 2006). Later, it was identified as an ubiquitination mediated proteasomal degradation of PCNA at K164 by the CUL4A E3 ligase. Phosphorylation of PCNA by active EGFR stabilizes PCNA by interfering with the interaction between PCNA and CUL4A (Lo, Ho et al. 2012).

The importance of Y211 residue of PCNA has recently been demonstrated in facilitating error-prone DNA replication by suppressing the MMR pathway (Ortega, Li et al. 2015). Inhibition of MMR at the very early steps occurs upon PCNA phosphorylation by EGFR. PCNA phosphorylation at Y211 interferes with the interaction between PCNA and MutSα and MutSβ (mismatch recognition proteins) and

abrogates PCNA-dependent activation of MutLα endonuclease and leads to nucleotide misincorporation during DNA synthesis.

### 1.6 Spatial distribution PCNA

PCNA exists in two pools within a cell, i.e. cytoplasm and nucleus. Nuclear PCNA functions are tightly linked to its ring-shaped structure, which allows PCNA to bind to numerous partner proteins to orchestrate DNA-related processes. Using single-particle tracking in combination with photoactivated-localization microscopy (sptPALM), the mobility of PCNA in live mammalian cells was studied and two populations of PCNA were found. Actively replicating cells showed slow diffusing PCNA located in replication foci and a fast diffusion nucleoplasmic pool of unbound PCNA which is not involved in DNA replication. The ratio of these two populations remained constant throughout different stages of S-phase (Zessin, Sporbert et al. 2016).

Neutrophil survival has been shown to be regulated by monomeric form of PCNA which is localized exclusively in the cytoplasm of neutrophils, due to its ability to be relocalized from nucleus to cytosol during granulocyte differentiation. Cytoplasmic PCNA exerts its anti-apoptotic activity in mature neutrophils independent of the trimeric conformation. In primary neutrophils, it has been shown that PCNA is associated constitutively with procaspase-3, procaspase-8, procaspase-9 and procaspase-10 to prevent their activation within the cytoplasm (De Chiara, Pederzoli-Ribeil et al. 2013). It has been shown that cyclin-dependent kinase inhibitor p21; compete with procaspases to bind PCNA which in turn triggers neutrophil apoptosis. PCNA acts as a regulator of neutrophil survival by providing a cytosolic platform through constitutive association with the procaspases and thereby preventing their activation and hence a decreased neutrophil apoptosis. This highlights a novel target to potentially modulate pathological inflammation (Witko-Sarsat, Mocek et al. 2010).

Moreover, PCNA is found to be associated with several cytoplasmic oncoproteins, including elongation factor, malate dehydrogenase, and peptidyl-prolyl isomerase.

Surprisingly, PCNA is also associated with six glycolytic enzymes that are involved in the regulation of steps 4-9 in the glycolysis pathway (Naryzhny and Lee 2010).

All these evidences suggests for a very diverse and versatile role of PCNA not only in nucleic acid metabolic pathways but also for various cellular functions.

### 1.7 Significance of Multiple PCNAs

With respect to PCNA, *Plasmodium falciparum* is one of the exceptions in having more than one PCNA, both of which have already been characterized (Kilbey, Fraser et al. 1993, Li, Warren et al. 2002, Patterson, Whittle et al. 2002). Others include the thermoacidophilic archaeon *Sulfolobus solfataricus* (De Felice, Sensen et al. 1999), the chlorella Virus (PBCV1) *Paramecium bursaria* (Lu, Li et al. 1995, Li, Lu et al. 1997), the Apicomplexa protozoa *Toxoplasma gondii* (Guerini, Que et al. 2000), the carrot plant *Daucus carota* (Hata, Kouchi et al. 1992), the maize plant *Zea mays* (Lopez, Khan et al. 1995, Lopez, Khan et al. 1997) and the plant *Arabidopsis thaliana* (Lin, Kaul et al. 1999, Theologis, Ecker et al. 2000).

In *Crenarchaea S. solfataricus*, PCNA is a heterotrimer of three distinct subunits (PCNA1, 2, and 3), in contrast to the homotrimer of eukaryotes. Distinct PCNA subunits assemble in a defined manner and possess specificity for binding to a particular protein at the lagging strand of the replicating fork. PCNA2 binds to the replicative PolB1, PCNA1 to FEN-1 and PCNA3 to DNA ligase I. These three factors have been shown to be bridged by PCNA *in vitro* (Dionne, Nookala et al. 2003). This provides a mechanism to tightly couple DNA synthesis and Okazaki fragment maturation. Additionally, unique subunit-specific interactions between components of the clamp loader, RFC, suggest a model for clamp loading of PCNA (Dionne, Nookala et al. 2003).

The genome of the *Euryarchaea*, *Thermococcus kodakaraensis* contains two genes encoding PCNA, making it unique among the Euryarchaea kingdom. It has been demonstrated that the two TkPCNA proteins form trimers and support processive DNA synthesis.

One of the notable differences between the TKPCNA trimers is that their interfaces are different, leading to different trimer stabilities (Ladner, Pan et al.).

Similarly, two distinct PCNA genes are expressed in *Daucus carota* (carrot plant) during somatic embryogenesis (Hata, Kouchi et al. 1992). One of the genes encodes for a conserved size PCNA (264 amino acids) while the other encodes a longer version (365 amino acids) with a molecular weight of 40.1 kDa suggesting it might form a PCNA dimer. The proliferating cell nuclear antigen (PCNA) gene family in *Zea mays* as well is composed of two homologues having similar expression profiles (Lopez, Khan et al. 1997).

*Arabidopsis thaliana* also possesses two homologues of PCNA. *in vitro and in vivo* studies revealed that PCNA2 specifically interacts with DNA pol λ and enhances its efficiency and fidelity in TLS (translesion synthesis) (Amoroso, Concia et al. 2011).

Crystal structures of the two *Arabidopsis thaliana* PCNAs (AtPCNAs) complexed with the C-terminal segment of human p21 were elucidated. The study showed that these two form heterotrimers that imply a critical role of hetero PCNA rings in cellular signal transduction, particularly in DNA repair (Strzalka, Oyama et al. 2009). With the aid of a bimolecular fluorescence complementation assay, the interaction was observed in the nucleus as well as in the cytoplasm, between PCNA1 and PCNA2. These findings indicate that PCNA1 and PCNA2 may cooperate in *A. thaliana* by the formation of both homotrimeric and heterotrimeric PCNA rings (Strzalka and Aggarwal 2013).

AtPCNA2 is supposed to be crucial for DNA polymerase η-dependent post-replication repair, but not AtPCNA1. Also, PCNA2 was shown to be necessary for DNA polymerase λ-dependent oxidative DNA damage bypass in Arabidopsis (Amoroso, Concia et al. 2011).

Both AtPCNAs were able to complement the yeast *POL30* (PCNA) mutant functionally, but failed to rescue the DNA damage tolerance defect of *pol30*. The two AtPCNAs have different levels of ubiquitination and sumoylation efficiency, leading to their differential responses upon DNA damage in yeast (Xue, Liang et al.

2015). AtPCNA2 has been shown to play a more critical role in DNA damage response as compared to AtPCNA1.

Apicomplexan parasites *Toxoplasma gondii* and *P. falciparum* both possess two genes encoding unique PCNAs (Kilbey, Fraser et al. 1993, Guerini, Que et al. 2000, Li, Warren et al. 2002, Patterson, Whittle et al. 2002). Phylogenetic analysis of the two apicomplexan PCNAs suggests the presence of more than one gene in their genome as a result of gene duplication. TgPCNA1, can complement a *POL30* cold-sensitive yeast strain against TgPCNA2; suggesting that TgPCNA1 may serve as a major processivity factor in *T. gondii* and is consistent with the failure to disrupt this gene in tachyzoites. Transgenic parasites in which the TgPCNA2 gene was disrupted showed no difference in DNA polymerase activity however, a slow growth rate was observed i*n vitro* (Guerini, Behnke et al. 2005).

**1.8 PfPCNAs**

Two homologues of PCNA are identified in *P. falciparum*. The gene encoding proliferating cell nuclear antigen, PCNA homologue is located on chromosome 13 and the coding sequence is 825 bases long that predicts for a 30.586 kDa protein. No introns were present and northern analysis reveals a transcript of ~1.6 kb. The characteristic residues of PCNAs of various eukaryotes including *Saccharomyces, Drosophila,* human and *Xenopus* were found to be conserved in PfPCNA; but the overall identity of PfPCNA with human (34%) and yeast PCNAs (31%) is low. PfPCNA1 is longer at its carboxy terminus (by 16 amino acids) than the PCNAs of other species. Its expression increases dramatically in late trophozoites and is maintained during the schizont stage (Kilbey, Fraser et al. 1993).

Additional proliferating cell nuclear antigen gene (PfPCNA2) in *Plasmodium falciparum* was identified to be located on chromosome 12 and encodes for a 264 amino acid long protein with a predicted molecular mass of 30.163 kDa. It shares only 29% identity and 53% similarity with PfPCNA1 at the protein level (Li, Warren et al. 2002, Patterson, Whittle et al. 2002). Northern blot analyses revealed the presence of two different

transcripts of *Pfpcna2*. One of the transcripts is expressed in both asexual and sexual stages of the intraerythrocytic cycle, while the other is present only in the sexual stage. This implies for multiple roles of PfPCNA2 in DNA metabolism in various stages of the parasite (Li, Warren et al. 2002). Another study indicated structural conservation of PfPCNA2 compared to eukaryotic PCNAs. PfPCNA (1 & 2) proteins showed differential expression in the asexual stages of the *P. falciparum* parasite. Expression of PfPCNA1 increases slowly about three-fold from ring to the late schizont stage. In contrast, robust increase in expression of PfPCNA2 protein level occurs from trophozoite and early schizonts which drops suddenly in late schizonts, suggesting that the two PfPCNAs may function under different physiological conditions (Patterson, Whittle et al. 2002).

Previous studies from our laboratory on PfPCNAs have established that PfPCNA1 could be the main processivity factor for the parasite because of its ability to successfully complement *ScPCNA* mutant strain in which genomic copy of *ScPCNA* has been deleted. However, *PfPCNA2* could not complement the *ScPCNA* mutant strain (Mitra, Banu et al. 2015).

Earlier DNA damage studies carried out in our laboratory involving PfPCNA1 have shown that in response to genotoxic agents like MMS (an alkylating agent) and HU (a Ribonucleotide reductase inhibitor), the protein undergoes up-regulation which possibly takes place at the protein level since the transcript level remains constant during the process. It has also been observed that the upregulated PfPCNA1 following DNA damage is enriched on the chromatin (how this transition from the nuclear soluble fraction to nuclear insoluble fraction takes place still remains elusive). Moreover, Wang et al., have shown phosphorylation of Tyr211 to be essential for chromatin loading of PCNA and its role in cell survival (Wang, Nakajima et al. 2006). Clustal W alignment of various PCNAs including *P. falciparum* (PfPCNA1 and PCNA2), *A. thaliana* (AtPCNA1 and PCNA2), Human, *S. cerevisiae* and *T. gondii* (TgPCNA1 and PCNA2) shows a conserved tyrosine (Y) residue at 213 and 210 positions of PfPCNA1 and PfPCNA2, respectively. Further, a recent phosphopreoteome data of schizont stage parasites

indicate the presence of a phosphorylated Ser residue at 191 position of PfPCNA1 but not in PfPCNA2 (Treeck, Sanders et al.). This study failed to report phosphorylation of conserved Tyr residue.

PfPCNA1 has recently been reported to be a component of a novel sensing system by which parasites respond to external stimuli including inflammatory cytokines, such as TNF. The study speculated HSP90 to be a component of a membrane-bound histidine kinase that receives the input stimuli and phosphorylated form of PfPCNA1 (triple phosphorylation) could be a response regulator. This sensing response could be regulated by a parasite checkpoint system and analogous to the bacterial two-component signal transduction systems (Wu, Cruz et al. 2016). Another study showed PfPCNA2 to be phosphorylated at S203 residue in merozoites only, wherein extensive differential protein phosphorylation was observed when intra-erythrocytic *P. falciparum* schizonts develop into extracellular invasive merozoites (Lasonder, Green et al. 2015).

All these studies indicate for a differential role of PfPCNAs in parasite biology. Therefore, it is interesting to know how the two PCNAs co-ordinate with each other for their respective functions in DNA metabolic pathways. Our study attempted to understand the regulation of PfPCNAs in DNA replication and vulnerability of the PfPCNAs to DNA damaging agents as these two proteins may share these functions in the parasite.

**Scope and objectives of the study**

Based on the above review of literature, we have tried to answer questions relevant to our study with the use of several cell and molecular biology techniques such as western blotting, Immunoprecipitation (IP), Co-immunoprecipitation (Co-IP), Fluorescence and Confocal microscopy and*in vitro* survival experiments to functionally characterize the two homologues of PfPCNA proteins i.e. PfPCNA1 (PF3D7_1361900) and PfPCNA2 (PF3D7_1226600) in DNA replication and DNA damage response. Since, the parasite possesses an ability to adapt to its host's cellular environment and to replicate its massive genome in a short span of time and overcoming the effects which may lead to genome

instability, studying mechanisms involved in DNA replication and DNA damage response with respect to the role of PfPCNAs would be useful in the field of parasite biology. Keeping the above facts and discussion in our mind, we designed the following study with the below mentioned objectives:

1. Comparative study of PCNA1 and PCNA2 in *P. falciparum* in DNA replication and DNA damage response
2. Functional relevance of the Proliferating cell nuclear antigens (PCNAs) of *P. falciparum* in DNA damage response
3. Implication of phosphorylation of PfPCNAs with respect to DNA replication and damage response

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# Materials and Methods

# MATERIALS

## Chemicals, Enzymes and Media

Restriction enzymes and DNA polymerase enzymes such as Taq polymerase and Phusion polymerase used for DNA manipulations were purchased from New England Biolab (NEB). The molecular biology grade chemicals were purchased from Sigma Aldrich (USA), Merck, Qualigen and SRL. PCR purification kit and Plasmid mini-preparation kits were obtained from Real Genomics or MDI. Plasmid Midi-Kit was purchased from Invitrogen. IPTG was purchased from G-Biosciences. Ni-NTA Agarose beads were procured from Qiagen; Amylose resin from NEB and Glutathione sepharose beads from Amersham Biosciences. Snake skin used for dialysis was purchased from Thermo-Pierce.

4,6 Diamidino-2-Phenylindole, Dihydrochloride (DAPI) used as a nuclear stain and antifade were procured from Invitrogen, Life technologies. Complete and incomplete Freund's adjuvants were purchased from Sigma-Aldrich, USA. PVDF membrane was purchased from MDI. Gel filtration column was procured from Amersham Biosciences (Pharmacia). LB (Luria-Bertani) Broth and LB Agar were provided by Hi-media (India). Protein molecular weight ladder was purchased from Fermentas and Thermo Scientific. For RNA work, Diethylpyrocarbonate (DEPC) was purchased from Sigma-Aldrich, USA; TRIzol for RNA extraction was obtained from Invitrogen. DNaseI enzyme as well as the reverse transcriptase enzyme (Verso cDNA synthesis kit) were obtained from Thermo Scientific.

Mass spectrometry grade milli Q, chemicals like CHAPS, DTT, IAA, Sodium thiosulphate, Sodium bicarbonate and Mineral oil were procured from Biorad, Sigma or Himedia. IPG strips for isoelectric focusing were obtained from Amersham Biosciences (Pharmacia).

For *P. falciparum* cell culture, RPMI 1640, Albumax$^{TM}$, Sodium bicarbonate and Hypoxanthine were procured from Invitrogen, Life technologies and Sigma Aldrich (USA). Geimsa stain for parasite infected RBC; Saponin for lysis of parasite infected

erythrocytes; Immersion oil for oil immersion lenses to visualize Geimsa stained parasites; Histopaque and other molecular biology grade chemicals were purchased from Sigma Aldrich (USA).

**Antibiotics and drugs**

Common antibiotics (Kanamycin, Ampicillin, and Chloramphenicol) used for bacterial culture were obtained from Hi-media (India). For *P. falciparum* parasite culture, Gentamycin was obtained from Sigma Aldrich. Hydroxyurea (Sigma), used for DNA damage experiments was prepared fresh in MQ (1M) and a final concentration of 10mM or 2.5 mM was used in the culture. Similarly, Methyl Methane Sulfonate (MMS) purchased from Sigma was used at working concentration of 0.005% or 0.002% in Complete RPMI (C.RPMI).

**Bacterial strains and Cloning/Expression Vectors**

Bacterial strains like *E. coli* DH10β and BL21-Codon plus (DE3)-RIL were used for cloning and expression of gene, respectively. The details of the above strains are provided in the following table M1.

**Table M1. List of bacterial strains used in this study**

| Strain/plasmid | Genotype/relevant characteristics | References/ Source |
|---|---|---|
| ***Escherichia coli* DH10β** | F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K - *rps*L (Str$^R$) *nup*G | Molecular cloning, Sambrook, *et al.*, |
| ***Escherichia coli* BL21-Codon Plus (DE3)-RIL** | *E. coli* B F$^-$ *ompT hsdS* ($r_B^-$ $m_B^-$) *dcm*$^+$ Tet$^r$ *gal* λ (DE3) *endA* Hte [*argU ileY leuW* Cam$^r$] | Novagen |

**Cloning/expression vectors, Recombinant Plasmid Constructs and primers**

The list of plasmids and recombinant plasmid constructs made/used in the present study with their relevant characteristics and source/reference where they have been utilized is given in table M2. The primers used for cloning or RT PCR amplifications are procured from either Eurofins or IDT and are listed in the Table M3.

**Table M2. The list of plasmids and recombinant plasmid constructs made/used**

| pDNA / recombinant pDNA | Charactereistics | Reference / Source |
|---|---|---|
| **pET28a** | *T7, His (N ter), Kan$^R$* | Novagen, Madison, WI, USA |
| **pET21c** | *T7, His (C ter), Amp$^R$* | A kind gift from Dr Rajesh Gokhale |
| **pMAL$_{c2x}$** | lacI, tac, malE, lacZα, bla, Amp$^R$ | Chapter 3 / RINGO (Kind gift from Dr. C. Doerig) |
| **pARL** | GFP vector | Kind gift from Dr. T. W. Gilberger |
| **pET-21c PfPCNA1** | pET-21c, C-His tag / NheI-XhoI | Chapter 1, 3 |
| **pET-28a PfPCNA2** | pET-28a,N-His tag /BamHI-XhoI | Chapter 1, 3 |
| **pET28aPfRad51 (largest ORF excluding the intron)** | pET28a N-His tag /BamHI-XhoI | Chapter 1 |
| **pET-28a PfERK8 (Kinase domain)** | pET-28a,N-His tag /BamHI-XhoI | Chapter 3 |
| **pET-21c PfPK5** | pET-21c, C-His tag / NheI-XhoI | Chapter 3 |
| **PfPCNA1 GFP** | GFP pARL vector /KpnI-AvrII | Chapter 1, 2, 3 |
| **PfPCNA2 GFP** | GFP pARL vector /KpnI-AvrII | Chapter 1, 2, 3 |
| **Y213F PfPCNA1 GFP** | GFP pARL vector /KpnI-AvrII | Chapter 3 |
| **S191A PfPCNA1 GFP** | GFP pARL vector /KpnI-AvrII | Chapter 3 |

| | | |
|---|---|---|
| **Y213F S191A (DM) PfPCNA1 GFP** | GFP pARL vector /KpnI-AvrII | Chapter 3 |
| **Y210F PfPCNA2 GFP** | GFP pARL vector /KpnI-AvrII | Chapter 3 |
| **PfPCNA1 Y213F pET-21c** | pET-21c, C-His tag / NheI-XhoI | Chapter 3 |
| **PfPCNA1 S191A pET-21c** | pET-21c, C-His tag / NheI-XhoI | Chapter 3 |
| **PfPCNA1DM (Y213F S191A) pET-21c** | pET-21c, C-His tag / NheI-XhoI | Chapter 3 |
| **pET-30 PfPCNA2 Y210F** | pET-30 EK/LIC, N-His tag | Chapter 3 |

**Table M3. List of Oligonucleotides used in this study**

| Name of the primer | Primer sequence | Use |
|---|---|---|
| **PfPCNA1 Fw 394** | 5'CGGGATCCGTGAAGAAGGATTTGATGCAG3' | RT PCR |
| **PCNA1 Rv 517** | 5' AGTAAATTTGATACAGTTAGA 3' | RT PCR |
| **PfPCNA2 Fw 385** | 5'CGGGATCCGAACATTTAGAAATACCACAATC 3' | RT PCR |
| **PCNA2 Rv 505** | 5' TTTCATAGATATAGAGACATT 3' | RT PCR |
| **PfRad51 Fw 702** | 5'CGGGATCCCGCTATGATGGCAGATGC 3' | RT PCR |
| **PfPCNA1 Y213F Fw** | 5'TCCTTTGCCATCAAATTTTTAAACTTGTTTTCT 3' | SDM |
| **PfPCNA1 Y213F Rv** | 5'AGAAAACAAGTTTAAAAATTTGATGGCAAAGGA 3' | SDM |
| **PfPCNA1 S191A Fw** | 5'CCAAGAGATTCAACTGCCGAAGATGATATTGGA 3' | SDM |
| **PfPCNA1 S191A Rv** | 5'TCCAATATCATCTTCGGCAGTTGAATCTCTTGG 3' | SDM |

| | | |
|---|---|---|
| **PfPCNA2 Y210F Fw** | 5'GAATTTGCTACAAGATTTCTAGTCA TGTTCTCA 3' | SDM |
| **PfPCNA2 Y210F Rv** | 5'TGAGAACATGACTAGAAATCTTGT AGCAAATTC 3' | SDM |
| **PfERK8 Fw (BamH1)** | 5'CGGGATCCATGCCTAAAGAAGATT GCA 3' | Cloning |
| **PfERK8 Rv (Xho1)** | 5'CCGCTCGAGTCATTCCCCTTGGTTC AATACATTA 3' | Cloning |

**Antibodies**

Antibodies used in this study were either generated using recombinant purified antigen (as described in the Methods section) or procured commercially, and are listed in Table M4.

**Table M4. List of Antibodies used in this study**

| Name of the Antibody used | Source | Remarks |
|---|---|---|
| **PfPCNA1** | Generated in Rabbit | 1:4000 (WB) / 1:2000 (IFA) |
| **PfPCNA2** | Generated in Mice | 1:3000 / 1:1500 (IFA) |
| **PfRad51** | Generated in Mice | 1:3000 (WB) |
| **Anti-PfActin (mice)** | Generated in our laboratory | 1:3000 (WB) |
| **Anti-$His_6$ antibody (Mouse)** | Abcam | 1:10000 (WB) |
| **Anti-GFP (Rabbit polyclonal)** | Abcam | 1:5000 (WB) |
| **Secondary Mouse IgG HRP** | Santa Cruz | 1:5000 (WB) |
| **Secondary Rabbit IgG HRP** | Santa Cruz | 1:5000 (WB) |
| **Alexa Flour 488 (Rabbit)** | Molecular Probes | 1:1500 (IFA) |
| **Alexa Flour 594 (Mice)** | Molecular Probes | 1:1500 (IFA) |

# METHODS

## DNA manipulations

### 1. Cloning of kinase domain of PfERK8 in pET28a Vector:

PfERK8 (PF3D7_1431500 MAP1) kinase domain (1-1098 nt) was PCR amplified using specific primers and cloned into the BamHI-XhoI site of pET-28a vector. DH10β cells were transformed with the recombinant constructs and the colonies were screened for the recombinant clones by colony PCR followed by clone confirmation by restriction digestion. All the selected clones were sequenced later (at Lab India sequencing facility).

### 2. Site-directed mutagenesis (SDM)

Point mutations were generated in PfPCNA1 and PfPCNA2 at conserved tyrosine residue (Y213 and Y210, respectively) and serine residue (S191, PfPCNA1) in pARL vector. Specific primers (Table M3) were used where tyrosine was converted into phenylalanine, and serine was converted into alanine. Site-directed mutagenesis was carried out by invert PCR method. After PCR, amplified products were digested with DpnI restriction enzyme at $37^0$C for 1 hr. DH10β competent cells were transformed with the digested products. DNA sequencing was performed to confirm for desired mutations in the ORF of the genes. PfPCNA1 double mutant was created using PfPCNA1Y213F mutant as template and using specific primers for the S191A in a similar way as described above.

## Recombinant protein purification ($His_6$-tagged fusion proteins)

$His_6$-tagged fusion proteins (WT PfPCNA1, Y213F PCNA1, S191A PCNA1 DM PCNA1, WT PfPCNA2, Y210F PCNA2, PfRAD51, PfERK and PfPK5) were expressed and purified by standard protocol as described by the manufacturer (Qiagen). *E. coli* BL21 (DE3) Codon plus competent cells were transformed with pET-21c/pET-28a-fusion constructs. Single colony was picked and grown in LB containing Chloramphenicol (25 μg $ml^{-1}$) or Ampicillin or Kanamycin (50 μg $ml^{-1}$). 1 liter LB was inoculated with 1% saturated culture and incubated at 37°C till $OD_{600}$ reached 0.6. The

culture was induced with 0.3 mM IPTG and further incubated at 22°C for 6-8 hours. After induction, cells were harvested. For the purification of recombinant protein, pellet was resuspended in 25-30 ml lysis buffer containing 20 mM Tris.Cl (pH 7.9), 300 mM NaCl, 10 mM Imidazole, 10 mg $ml^{-1}$ lysozyme and 100μM PMSF. Cell suspension was sonicated further and incubated at 4°C with Triton X-100 for 30 minutes. The lysate was centrifuged at 15000*g* for 30 minutes. The resulting supernatant containing soluble fraction was incubated with NiNTA beads at 4°C for 1 hour. After binding, beads were separated from unbound fraction of protein by centrifugation. The protein bound beads were washed with lysis buffer containing varying amounts of Imidazole to remove non-specifically bound proteins. Finally buffer containing 300-500 mM Imidazole was used to elute the bound proteins. The eluted protein was either used for biochemical assays or dialyzed against buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol, 100 mM NaCl and 100 μM PMSF. Dialyzed protein was stored at -80°C.

**Recombinant protein purification (MBP-tagged fusion proteins)**

MBP fusion protein (MBP-RINGO) was expressed and purified by a standard protocol as described by the manufacturer (New England Biolabs). *E. coli* BL21 (DE3) Codon plus competent cells were transformed with MBP fusion construct. LB was inoculated with 1% overnight grown primary culture in the presence of Ampicillin (50 μg/ml) and Chloroamphenicol (25 μg/ml) and was grown till $A_{600}$ reached 0.5-0.6. Culture was induced with 0.5 mM IPTG and incubated further for 8–10 hours at $22^0$C. Following induction, bacterial cells were harvested from 2 liter culture and re-suspended in 20 ml lysis buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 100 μM PMSF). Cell debris was removed by high-speed centrifugation and soluble fraction was incubated in the presence of pre-equilibrated 0.5 ml amylose resin at $4^0$C for 1 hour. Following incubation, resin was washed with the lysis buffer. Proteins were usually kept in the bead bound state for pull down assays; otherwise fusion protein could be eluted with lysis buffer containing 10 mM maltose. Bead bound proteins were stored at 4°C and were used as soon as possible.

**Size-Exclusion chromatography (26ml Superdex-200 10/300 GL)**

For purification and oligomerization of WT PCNAs and their mutant proteins, we used small size column 26 ml Superdex 200 10/300 GL (GE Healthcare, Uppsala, Sweden). $His_6$- WT PfPCNA1, Y213F PCNA1, S191A PCNA1 DM PCNA1, WT PfPCNA2 and Y210F PCNA2 mutant proteins were purified by 26 ml Gel filtration chromatography column. For this purpose, Ni-NTA agarose purified concentrated 0.5 ml protein mixture was loaded on calibrated 26 ml Gel filtration chromatography column. The proteins were centrifuged at 10000g for 10 minutes to remove the particulate impurities. The running buffer includes 10 mM Sodium cacodylate buffer (pH 7.4), 5 mM ßME and 100 µM PMSF. The column was calibrated using molecular weight standards as followed: Catalase (232 kDa), Aldolase (158 kDa), Bovine serum albumin (66 kDa), Oval albumin (43 kDa) and Chymotrypsinogen (25 kDa). A standard curve was obtained by plotting the molecular mass of the standard proteins (in logarithmic scale) against the elution volume using Graph-pad Prism curve fitting software. The flow rate in all determinations was 0.3 ml/min and fractions of 0.5 ml were collected. Each expected peak fractions were analyzed on SDS-PAGE.

**Polyclonal antibody production**

Polyclonal antibodies against PfPCNA1 (rabbit), PfPCNA2 (mice) and PfRad51 (mice) were raised using respective purified recombinant $His_6$-tag proteins. For this purpose, purified protein was resolved in 10% SDS-PAGE and the desired band was excised from the gel. Gel slice was chopped finely and further crushed by passing through a three way connector where the gel pieces were mixed with Complete Freunds adjuvant (for priming) (Sigma) and injected into rabbit/mice. For booster doses, gel pieces were mixed with Incomplete Freunds adjuvant (Sigma) for injections. Each mouse was primed with approximately 200 µg of immunogen followed by boosters, while rabbit was primed with approximately 500 µg of immunogen followed by boosters, at $14^{th}$ and $21^{st}$ day before the first immune sera was collected. Subsequently, several bleeds were collected with several booster doses interspersed between them. All the collected bleeds were tested appropriately and aliquots were stored at -80ºC and a working aliquot at

4ºC. In all the cases pre-immune sera was collected before priming the animal with the antigen and was tested for cross-reactivity with the antigen.

**Western blot analysis using recombinant protein or parasite lysate**

Purified recombinant protein, IPTG induced bacterial lysate, parasite lysate or immunoprecipitates were resolved in 10%, 12% or 15 % (depending on the protein to be identified in the immunoblot assay) SDS-PAGE. SDS-PAGE were prepared and run following standard SDS-PAGE protocol. Appropriately run gels were transferred using OWL semi-dry electroblotter (HEP-1) system onto PVDF membrane (Millipore/MDI technologies) using Transfer buffer containing 20% Methanol, Glycine, Tris base according to manufacturer's instructions. PVDF with transferred proteins was then blocked with 5% skimmed milk prepared in (1X) Phosphate buffered Saline (1XPBS) containing NaCl, $Na_2HPO_4$, $KH_2PO_4$ and KCl with addition of 0.1% Tween 20 detergent for washing purposes. After the blocking was washed off using 1XPBST, PVDF containing the transferred proteins was treated with desired primary antibody in appropriate dilution followed by washing in PBST and appropriate secondary antibody (HRP conjugated) treatment followed by washing and developing and visualization using chemiluminiscence based immunodetection using luminol, coumaric acid and hydrogen peroxide cocktail or ECL chemiluminiscent kit (Amersham, USA) using manufacturer's instruction with minor modifications. For each primary antibody, conditions of antibody treatment including dilution to be used and duration of the treatment was individually standardized.

***In vitro* phosphorylation with recombinant kinases**

Kinase assays were performed as previously described (Leykauf et al., 2010) in a standard reaction of 30 µl containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 0.1mM EDTA and 200µM ATP and 5 µCi of [$\gamma$-$^{32}$P] ATP (BRIT, Hyderabad). Each reaction contained 3 µg of recombinant PfERK8 or PfPK5 proteins (kinase), along with ~5 µg recombinant proteins (PfPCNA1, PfPCNA2, MBP or Histione H1) to be tested in the kinase reaction buffer. Reaction was performed at 30$^0$C for 30 minutes and

further terminated by the addition of SDS buffer. Samples were boiled at $95^0$C for 5 min and resolved in 12% SDS-PAGE. The gels were dried and subjected to autoradiography.

### *P. falciparum* genomic DNA isolation

*P. falciparum* infected erythrocytes were lysed with 0.015% saponin and washed with 1X PBS. Parasite pellet was re-suspended in lysis buffer (50 mM Tris, pH8.0/ 50 mM EDTA/100 mM NaCl) containing 2% SDS and Proteinase-K (100 μg/ml) and incubated at 37°C (overnight). Extraction was performed twice in phenol/chloroform (1:1) and once in chloroform. DNA was precipitated from the aqueous phase with 3 M sodium acetate ($1/10^{th}$ vol) and ice- cold absolute ethanol (2 vol) and allowed to incubate for 20 min at -20°C followed by centrifugation (12,000 x g) and washing the pellet with 70% ethanol. The pellet was air-dried and re-suspended in TE (10 mM Tris-HCl, pH 8.0/1 mM EDTA).

### RNA Isolation from *P. falciparum*

Total RNA was isolated from parasites treated with hydroxyurea where parasites were released from infected erythrocytes with 0.015% saponin. Trizol reagent (Invitrogen) was added to the parasite pellet and samples were incubated for 5 min followed by the addition of 200 μl of chloroform. Sample was vigorously mixed and incubated further for 2-3 minutes at room-temperature. Cells were centrifuged at 12,000 rpm for 15 minutes at 2-$8^0$C. After centrifugation, upper layer containing the RNA was carefully transferred to a fresh microfuge tube. RNA was precipitated from the aqueous phase by isopropanol. Pellet was washed with 70% ethanol. Finally, dried pellet was dissolved in Diethylpyrocarbonate (DEPC) treated or RNAase free water and incubated at $65^0$C for 4-5 minutes to facilitate dissolution of the pellet. RNA was quantitated spectrophotometrically. At this stage the total RNA isolated was either stored at-$80^0$C or proceeded for cDNA synthesis. The cDNA was prepared using protocol as described earlier (Mehra et al., 2005).

**RT-PCR analysis**

Isolated RNA was first subjected to DNaseI treatment (RNAse free, Invitrogen, RT PCR kits) followed by first strand cDNA synthesis. cDNA was synthesized with oligodT primer using ~5 µg of stage specific RNA and amplified by Verso cDNA reverse transcriptase (RT) (Invitrogen) in 30 µl of reaction mixture. 2 µl of the cDNA reaction was used to amplify the fragments of PfPCNA1, PfPCNA2, PfGAPDH and PfRad51. PCR amplified product was further resolved on 1.5 % agarose gel to visualize the amplification. All the primers are shown in table M3.

**Parasite Culture and synchronization**

*Plasmodium falciparum* strain 3D7 was cultured in human erythrocytes in RPMI 1640 medium supplemented with 0.2% $NaHCO_3$, 0.5% Albumax (Invitrogen), Gentamicin sulfate (10µg/ml), Hypoxanthine (27 mg/liter). Parasitaemia was maintained at 3-4% for routine culture. Parasite culture was supplied with mixed gas containing 90% Nitrogen, 5% Oxygen and maintained at 37°C (Trager and Jensen, 1976).

For synchronization of parasites, 5-6% parasitaemia was used. Ring stage parasites were treated with 5% sorbitol for 5-7 minutes at room temperature, followed by washing with incomplete RPMI twice and once with complete RPMI. Parasites (Geimsa stained) were checked routinely under microscope before harvesting. Infected erythrocytes were lysed using 0.015% saponin in 1X PBS and parasites were recovered after centrifugation (1000x g) and washed with cold 1X PBS.

**Treatment of parasite using genotoxic agents**

Parasites with 8-10 % parasitaemia and 4% hematocrit and synchronized to mid-trophozoite stage (90%) were used for treatment with genotoxic agents. Hydroxyurea or Methyl Methane Sulfonate treatment was done using a standard concentration of 10mM or 0.005%, respectively (unless mentioned otherwise) for a time period of 6 hours. At the end of the treatment in each case, Geimsa stained parasite smears were prepared to check for any morphological distortions. Parasites were retrieved and released from erythrocyte using 0.015% saponin prepared in 1X PBS, followed by washing 2-3 times in

1X PBS. Purified parasite was either subjected to WB analysis or RNA was extracted from the parasites for RT PCR analysis as described later.

**Stage specific western blot analysis from *Plasmodium* cell extract**

Asexual stage parasites from Ring, Trophozoite and Schizont stages synchronized to 90-95% synchrony were used for Stage specific Western blot analyses. The parasites were lysed with 0.1% saponin (Sigma-Aldrich) to remove erythrocyte membrane fraction from parasites, followed by washing with ice cold 1X PBS 2-3 times. The parasite pellet obtained was then boiled at 95°C for 5-8 minutes after suspending using 6X SDS gel loading buffer. Stage specific lysate was then resolved in 10 or 12% SDS-PAGE and transferred onto PVDF membrane. Western blot was performed using various antibodies, following standard protocol (Sambrook et al., 1989).

**Sub cellular Fractionation**

The nuclear soluble and chromatin bound fractions were prepared using a modified protocol described previously (Fleuck et al., 2009). Briefly, parasites were lysed in buffer A (20mM HEPES (pH 7.9), 10mMKCl, 1mM EDTA, 1mM EGTA, 0.3% NP-40, 1mMDTT,and protease inhibitor cocktail (Sigma) with incubation on ice for 10 minutes. Nuclei were pelleted down at 8000 rpm and the nuclear soluble fraction was saved. The pellet obtained was washed twice in buffer A followed by lysis in buffer B (20mM Tris-HCl pH7.5, 100mM NaCl, 60mM KCl, 1mM $CaCl_2$, 5mM $MgCl_2$, 5mM $MnCl_2$, 300mM sucrose, 0.4% NP40, 1mM DTT and protease inhibitor cocktail) for 45 minutes on ice. The debris recovered after centrifugation at 13000 rpm was saved as the chromatin fraction. The fractionation efficiency was assessed by antibodies against Histone H3 and PfHsp70.

**Co-immunoprecipitation from *Plasmodium falciparum* extract**

To investigate the interaction between PFPCNA1 and PfPCNA2, we performed co-immunoprecipitation experiment from the *Plasmodium falciparum* parasite extracts. For this purpose, asynchronous *P. falciparum* infected RBC (8-10% parasitaemia) were lysed with 0.1% saponin (Sigma-Aldrich) to remove erythrocyte membrane fraction from

parasites, followed by washing with ice cold 1X PBS 2-3 times. The parasite pellet obtained was then lysed using lysis buffer containing 50mM Tris-HCl, pH-8.0, 150mM NaCl, 2 mM EDTA, 0.5% NP-40 and Protease inhibitor cocktail for 1 hour at $4^0$C. The lysate was cleared by centrifugation at 12,000 rpm for 15 min at $4^0$C and quantitated. Approximately 1 mg parasite extract was taken for each immunoprecipitation reaction was incubated with preimmune sera or polyclonal antisera against either PfPCNA1 or PfPCNA2. The immune complexes were allowed to form by incubation at 4°C at 5-6 rpm overnight. After incubation, Protein sepharose A beads were incubated for 1 hr at 4° C at 5-6 rpm to pull down the immune complexes. Non-specific interactions were removed by washing using the same lysis buffer with higher NP-40 concentrations. The interacting proteins were released from the beads and antibodies by resuspending in 6XSDS gel loading buffer, followed by 12% SDS-PAGE and WB analysis using specific antibodies.

**Immunofluorescence and co- immunofluorescence Analysis**

Smears of *P. falciparum* infected erythrocytes from synchronized as well as mixed stages were prepared on Glass slides. Cells were dip fixed in chilled absolute methanol for few seconds followed by incubation for 1 hour at room temperature in blocking solution containing 3% BSA, 0.01% saponin in 1XPBS. The slides were then incubated either in the presence of immune sera raised against different *P. falciparum* proteins or with pre-immune sera, at 4°C for 1 hr. The slides were then washed with 1X PBST 4-5 times. In case of co- immunofluorescence, parasites were incubated with respective primary antibodies followed by washing with 1X PBST 4-5 times. Primary binding is then followed by incubation with secondary antibody (Alexa Flour 488 anti-rabbit IgG or Alexa Flour 594 anti-mice IgG) for 45 minutes at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole(DAPI, Invitrogen) at concentration of 1µg/ml followed by washing with 1X PBST and mounted in 90% glycerol containing 0.1% Paraphenylenyldiamine (PPD, Sigma) and further analysed with a Zeiss Imager.Z1(Apotome) using Axiovision 4.6.3 SP1 software.

**Parasite transfection and confocal microscopy**

Fresh erythrocytes were loaded with ~100µg of midi-kit purified Y213F PCNA1-pARL, S191A PCNA1-pARL, DM (Y213F S191A) PCNA1-pARL and Y210F PCNA2-pARL plasmid DNA followed by electroporation using Biorad Gene Pulser X Cell. To the loaded RBCs, highly enriched schizont stage parasites (~95%) were added and allowed to grow in the absence of drug through one erythrocytic cycle (48 hours) after which gradually increasing concentration of WR99210 drug (2-5 nM) was added to the culture medium. Subsequently, media was changed every day and the drug pressure was maintained. Western blot analysis and live cell imaging was done to confirm the presence og GFP signals using Zeiss Imager.Z1 (Apotome) using Axiovision 4.6.3 SP1 software.

**DNA damage and confocal microscopy**

Live cell imaging was done using PfPCNA1-GFP and PfPCNA2-GFP transfectants following treatment with 10 mM HU for 6 hr, using Olympus FV 1000 laser scanning confocal microscope. Nuclei were stained by incubating the cells with DAPI for 5 minutes at 37°C prior to imaging. GFP signals were measured in untreated and HU treated parasites using OLYMPUS FV1000 laser scanning confocal microscope. Olympus Fluoview FV10-ASW 4.0 software was used to quantitatively investigate the intensity of PCNA2-GFP signals in untreated and HU treated parasites. Distinct PCNA1-GFP foci were counted in untreated and HU treated parasites taking the aid of Z stack analysis tool. The data were plotted with mean and standard error.

***in vitro* parasite survival assay**

To check for survival of the parasites after treatment with the genotoxic agents, synchronized parasites at late trophozoite stage (~36hrs) was maintained at 1% parasitaemia and the parasites were treated with HU and MMS at a concentration of 2.5 mM and 0.002% (v/v), respectively for a time period of 6 hours. The dosage of DNA damaging agents was adjusted for these experiments as we found lack of parasite growth following treatment with 10 mM HU and 0.005% (v/v) MMS indicating that the parasites were under severe cellular stress at these drug concentrations. There might be

threshold of DNA damage beyond which the parasite repair machinery was overwhelmed and the parasites succumbed to the lethal effects of DNA damage. At the end of the treatment of 6 hrs incubation with genotoxic agents, parasites were washed once with incomplete RPMI media and resuspended in fresh complete RPMI media and allowed to grow further (~26 hours) to the next cycle till the parasites reached late ring stage. Giemsa stained slides were then prepared and parasitemia was counted. A total of 800 cells were counted from three independent experiments. The graphs were plotted for fold increase in parasitemia in untreated versus genotoxic agent treated parasites with mean and standard error. Further statistical analysis (Student's *t* test) was also performed as detailed below.

**Statistical Analysis**

Western blot band intensities were analyzed densitometrically by the Image J software. Data were analyzed and reported as the mean and SEM for independent experiments (n=3). Statistical analyses were done using Microsoft Excel and GraphPad Prism. Differences between means were determined using Student's *t* test, and $p \leq 0.05$ was considered significant. Student's *t* test was also performed for PfPCNA1 foci calculation (before and after DNA damage), and PfPCNA2 nuclear enrichment following DNA damage and for the *in vitro* parasite survival assay.

**CD spectroscopy**

WT PfPCNA1 & 2 and their mutant forms (Y213F PCNA1, S191A PCNA1, DM PCNA1, and Y210F PCNA2) were mixed in 10 mM Sodium cacodylate buffer, pH 7.4 at a final concentration of 0.1 mg/ml. All measurements were carried out at $25^0$C. CD spectra were recorded on a Chirascan Applied photophysics U.K. using cells containing 0.1 cm path length. Spectral data were acquired over the range 260–190 nm for the far-UV CD measurement. Ellipticity values (θ) in mdeg, obtained from the instrument, were expressed in terms of mean residue ellipticity [θ] using the following equation:

$$[\theta] = \theta\ M/10XL$$

Where, M is the mean residue mass, L the path length (0.1 cm), C is the concentration in g/ml and θ is the mean residue ellipticity. CD data were further processed using K2d2 software for the prediction of secondary structure.

**Two-dimensional Gel Electrophoresis**

Synchronized parasites treated with 10 mM HU in late trophozoite stage (~36 hrs) were saponin lysed and washed with 1XPBST to remove RBC membrane completely. The parasites were lysed using lysis buffer containing 50mM Tris-HCl, pH-8.0, 150mM NaCl, 2 mM EDTA, 0.5% NP-40 and Protease inhibitor cocktail for 1 hour at $4^0$C. The lysate was cleared by centrifugation at 12,000 rpm for 15 min at $4^0$C and quantitated. Approximately 400 µg protein extract of each of the untreated and HU treated parasites was taken for sample preparation for Isoelectric focusing. Proteins samples were precipitated by TCA (1:8:1:: cell lysate: 100% ice-cold acetone : 100% trichloroacetic acid). Following this, the protein was resuspended in 300 µl of Urea-Thiourea rehydration buffer (7Murea, 2 M Thiourea, 2% CHAPS, 65 mM DTT, 1% IPG buffer, pH 3–10, 0.001% bromphenol blue). Protein sample was centrifuged to settle down particulate material and ~250 µl is then applied to a 13 cm immobiline pH gradient Dry Strip (3-10, pH; Amersham Biosciences). IPG gel strips were rehydrated overnight at 20°C in the Immobiline DryStrip Reswelling Tray (Amersham Biosciences). Isoelectric focusing (IEF) was run on IPG phor IEF Unit (Amersham). The running programme consists of 2 h for 100 V, 2 h for 500 V, 2 h for 1000 V, 8000 - 20,000 Vh, 8 h for 8,000 V and finally 10h at 3000 V. The voltage was increased gradually until a total of 60,000 V was reached. The focused strips were equilibrated in 10 ml equilibration solution (75 mM Tris-HCl, pH 8.8, 6 M urea, 29.3 % glycerol (v/v), 2% SDS, 0.002% BPB) with reducing agent of 1% DTT for 15 min, and 10 ml equilibration solution with 4.5% iodoacetamide for another 15 min. The strips were then washed twice briefly with 1× SDS gel running buffer and loaded on 10 or 12.5% SDS-PAGE gels for second dimension separation. The gels were run at constant current 40 mA in a Laemmli's buffer system until the dye front reached the bottom of the gel.

**Protein detection by Silver staining, MALDI TOF mass spectrometry for protein identification**

Proteins in the gel were "silver stained" by fixing the gel in Fixer solution (50%MeOH and 10% acetic acid) overnight followed by washing in 30 % Ethanol for 10 min. The gel was then rinsed with water (MQ) for 10 min and then treated with sodium thiosulfate (0.2 g/liter) for 1 min. After rinsing twice in water (60 s for each time), the gel was incubated in silver nitrate (2.0 g/liter) for 10 min. After washing once with water (20 s) and once for 30 s with Developing Solution (sodium carbonate, 30 g/liter, formaldehyde, 1.4 ml of 37% solution/liter, and sodium thiosulfate, 10 mg/liter), protein bands/spots were visualized by further treating the gel with Developing Solution. The reaction was stopped with 6% acetic acid solution. Finally, the gel was analysed for the changes in protein profile. Upregulated protein spots were excised manually using fresh scalpels and proceeded for standard in gel trypsin digestion protocol for further mass spectrometric analysis for protein identification. Digested peptides were analyzed by MALDI TOF. Protein identification was performed by sending trypsin digested peptide masses to the *P. falciparum* databases of National Centre for Biotechnology Information (NCBI) using the MASCOT (Matrix Science) Peptide Mass Fingerprinting programme.

# Comparative study of PCNA1 and PCNA2 in *P. falciparum* in DNA replication and DNA damage response

*P. falciparum* has a complicated life cycle and DNA replication process distinct from other model systems. Proliferating cell nuclear antigen is one of the key molecules of DNA replication machinery as it forms a homotrimeric ring around the DNA strands and primarily acts as the processivity factors. Although PCNA is primarily the processivity factor for DNA polymerases to aid in elongating the newly synthesizing DNA, the protein is well documented for its versatility as it plays important role in other cellular processes including DNA repair, translesion DNA synthesis, DNA methylation, chromatin remodeling, gene expression, cell cycle regulation and prevention of apoptosis. *P. falciparum* possesses two homologues of PCNAs unlike that of most other eukaryotes. Present study focuses on comparative study of PfPCNAs in DNA replication and DNA damage response pathways.

The amino acid sequence of PfPCNA1 is ~30% identical to the related proteins from other eukaryotes (Kilbey, Fraser et al. 1993) and the alternate copy of PCNA (PfPCNA2) shows only 23% identity to PfPCNA1 (Patterson, Whittle et al. 2002). PfPCNA1 and 2 show differential expression in the intra-erythrocytic cell cycle of the malaria parasite. Stage specific expression of PfPCNA1 reveals the expression of this protein is higher in trophozoite and schizont stages (i.e. during and post replication). The expression slowly increases about threefold from the ring to the late schizont stage. In contrast, PfPCNA2 shows robust expression in trophozoites and early schizonts with a sudden drop in expression in the late schizont stage (Patterson, Whittle et al. 2002).

Previous studies from our laboratory on PfPCNAs established that PfPCNA1 could be the main processivity factor for the parasite because of its ability to successfully complement *Scpcna* mutant strain in which the genomic copy of *Scpcna* has been deleted. However, PfPCNA2 could not complement the *Scpcna* mutant strain (Mitra, Banu et al. 2015). Moreover, PfPCNA1 has been shown to be a component of replication factories termed as replisome since endogenous as well as over-expressed PfPCNA1 GFP fusion protein form punctate foci in the parasites co-localizing with PfORC5 (Gupta, Mehra et al. 2008). However, over-expressed PfPCNA2 GFP fusion protein shows a diffused distribution pattern within the cytoplasm and nucleus during and after the replicating stages.

With respect to PfPCNAs, our laboratory is particularly interested in studying their role in DNA damage pathways. Earlier studies carried out in our laboratory involving PfPCNA1 have shown that in response to genotoxic agents like MMS (an alkylating agent) and HU (a Ribonucleotide reductase inhibitor); the protein undergoes up-regulation which is post-transcriptionally regulated. However, the question remained unanswered for the role of alternate processivity factor of *Plasmodium falciparum* i.e. PfPCNA2 in DNA replication and DNA damage response due to the lack of specific antibodies against PfPCNA2. Therefore, it will be interesting to know how the two PCNAs of *P. falciparum* co-ordinate with each other for their respective functions in DNA metabolic pathways. In order to understand the role of PfPCNAs in DNA replication and DNA damage response. We asked several questions that include whether PfPCNA2 undergoes similar up-regulation like PfPCNA1 at the protein level in response to DNA damaging conditions using genotoxic agents like MMS (BER pathway) and HU (Ribonucleotide reductase inhibitor). Also, whether PfPCNAs behave similar to other DNA damaging agents like ionizing radiations causing Double strand breaks (DSBs) and UV irradiation (NER pathways) as observed with MMS and HU for their significance in different repair pathways.

## RESULTS

### Polyclonal antibody generation against the PfPCNAs

To study the comparative role of PfPCNAs in DNA replication and DNA damage response, specific antibodies are required in two different hosts. For this purpose, recombinant full length PfPCNA1-$His_6$ and $His_6$-PfPCNA2 proteins were affinity purified using Ni-NTA beads from *E. coli* BL21 codon plus cells and injected in rabbit and mice, respectively as per the protocol described in materials and methods section. The full length PfPCNA1 and PfPCNA2 were cloned by Dr. Pallabi Mitra in pET21c (+) and pET28a (+) vectors, respectively. The specificity of the immune sera was checked by western blot experiments using purified protein as well as *P. falciparum* 3D7 parasite lysate. Specific bands at ~30 KDa were obtained (for recombinant protein as well as in parasite lysate) for both the PfPCNAs (Figure 1.1, 1.2). However, no signals were observed when pre-immune sera were used for western blot experiment under the same experimental conditions. The antibodies

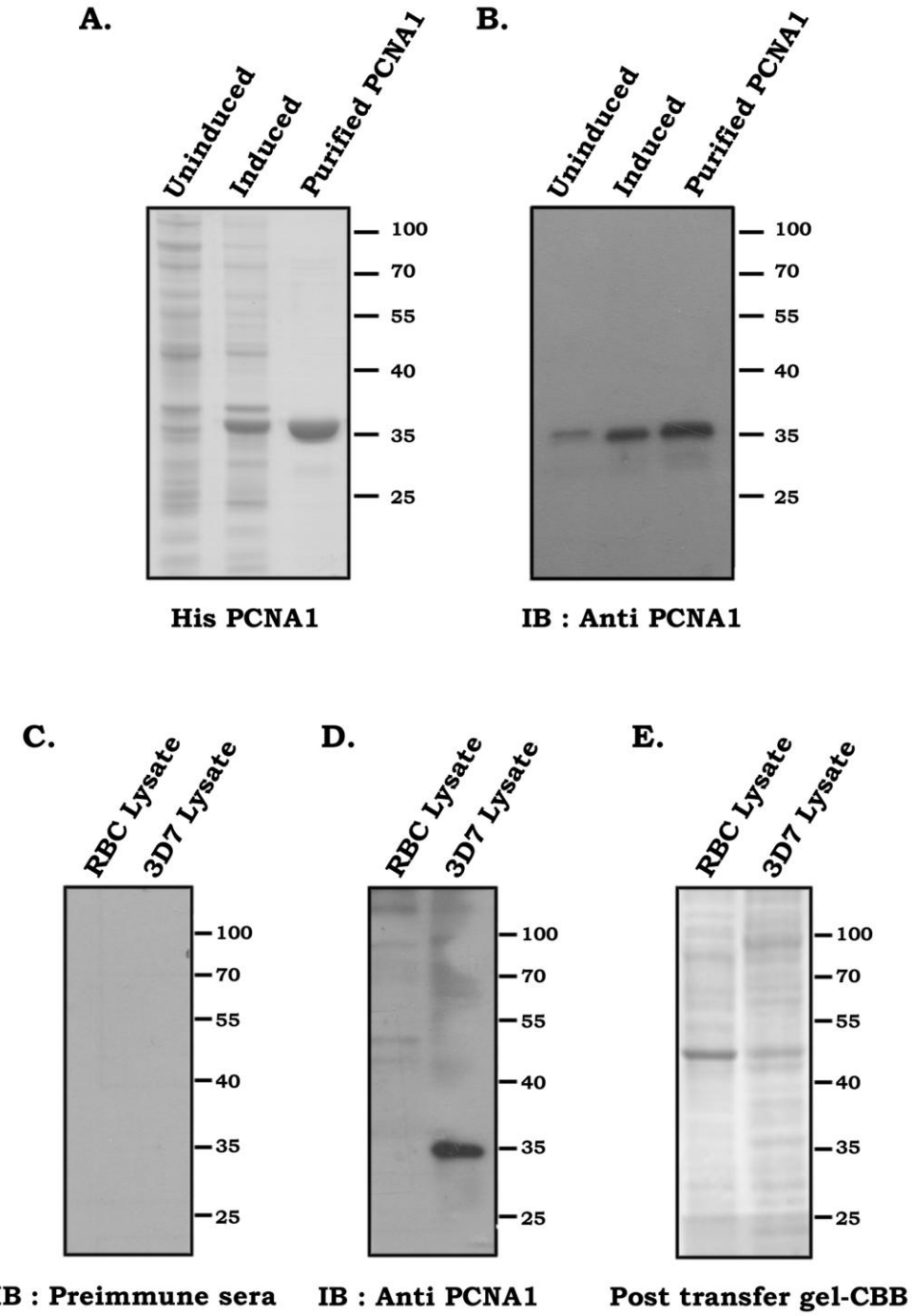


**Figure 1.1. Purification profile of $His_6$-tagged PfPCNA1 protein and Western blotting to show the specificity of PfPCNA1 antibodies. (A)** Purification profile of $His_6$ PfPCNA1. Coomassie stained gel shows the induction of PfPCNA1 protein in *E. coli* BL21-DE3 cells as well as the purified protein after Ni-NTA affinity purification. **(B)** Western blot analysis of the protein using antibodies against PfPCNA1 as shown in (A). **(C-D)** The expression of endogenous PfPCNA1 was checked by pre-immune sera or antibodies against PfPCNA1 in *P. falciparum* lysate or RBC lysate. A single band was observed with immune sera but not in the presence of pre-immune sera. **(E)** Coomassie stained gel after transfer to show the loading control.

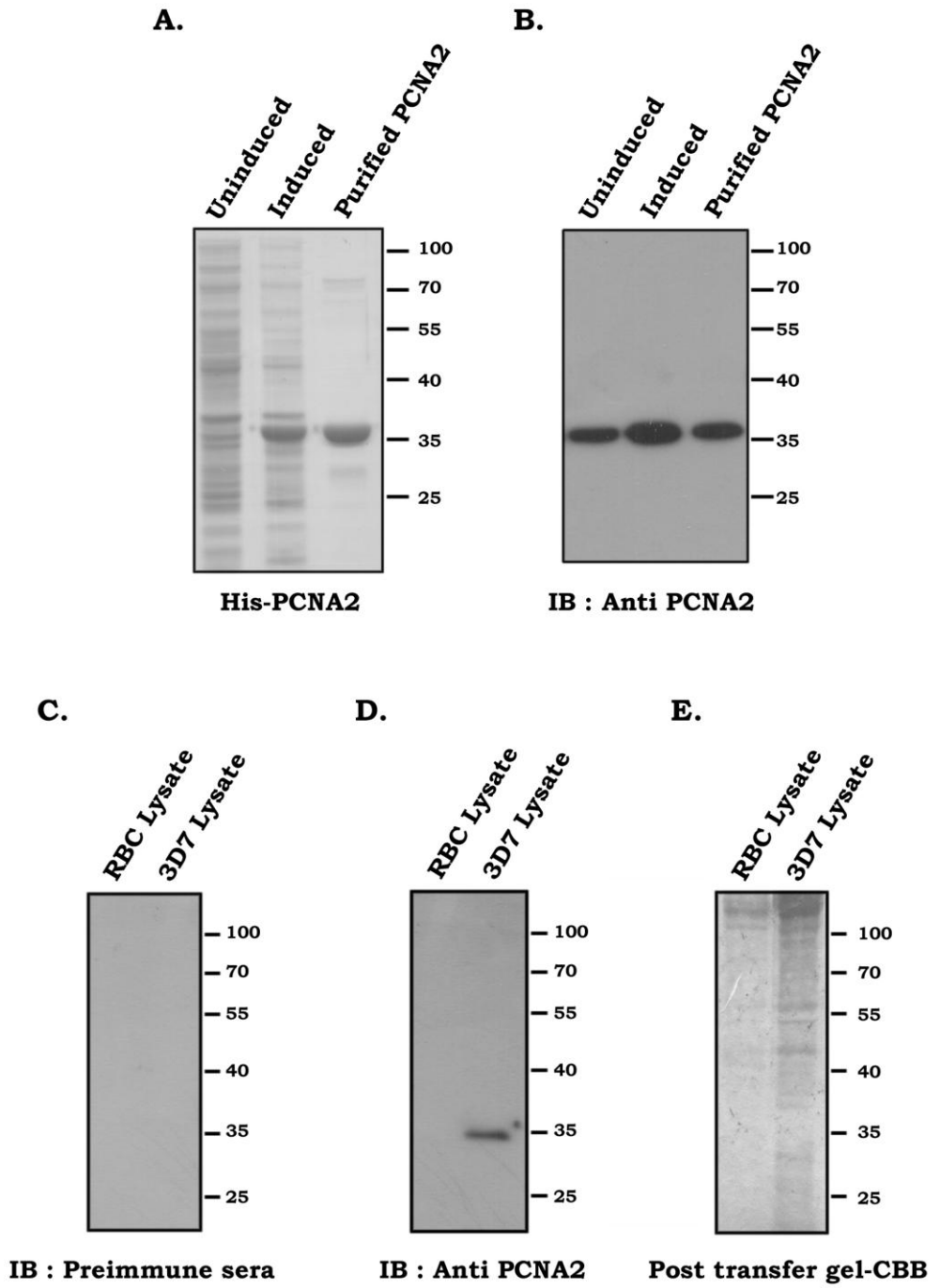


**Figure 1.2. Purification profile of $His_6$-tagged PfPCNA2 protein and Western blotting to show the specificity of PfPCNA2 antibodies. (A)** Purification profile of $His_6$ PfPCNA2. Coomassie stained gel shows the induction of PfPCNA2 protein in *E. coli* BL21-DE3 cells as well as the purified protein after Ni-NTA affinity purification. **(B)** Western blot analysis of the protein using antibodies against PfPCNA2 as shown in (A). **(C-D)** The expression of endogenous PfPCNA2 was checked by pre-immune sera or antibodies against PfPCNA2 in *P. falciparum* lysate or RBC lysate. A single band was observed with immune sera but not in the presence of pre-immune sera. **(E)** Coomassie stained gel after transfer to show the loading control.

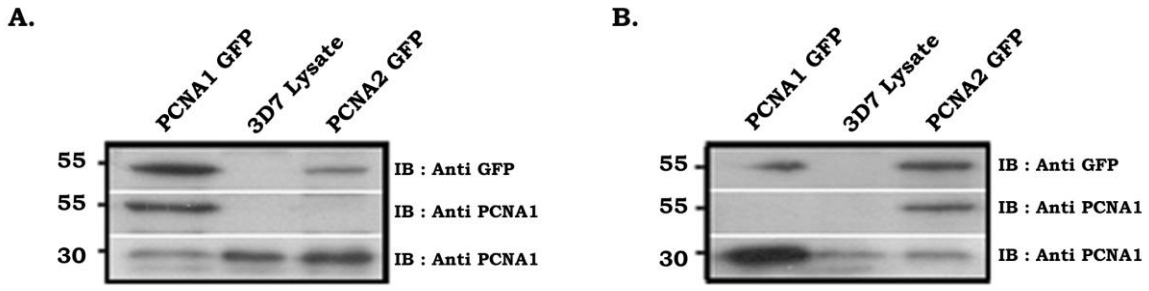


**Figure 1.3. (A-B) Antibodies against PCNA1 and PCNA2 recognize the respective proteins.** Cross-reactivity of PfPCNA1 and PfPCNA2 antibodies were checked by Western blot analysis using parasite lysate obtained from 3D7, PfPCNA1-GFP and PfPCNA2-GFP expressing parasites. Antibodies against PfPCNA1 specifically identified the endogenous protein band in all the lanes while PfPCNA1-GFP protein was exclusively recognized in PCNA1-GFP expressing parasites. Antibodies against PfPCNA2 also showed specificity in the similar manner. GFP antibodies confirmed the expression of GFP-fusion proteins in the parasites.

against PfPCNA1 and PfPCNA2 were subsequently tested for their cross-reactivity with endogenous parasite proteins in *P. falciparum* 3D7 parasites and parasites over-expressing PCNA1-GFP and PCNA2-GFP (Figure 1.3). The antibodies specific for PCNA1 recognized a specific band for endogenous PCNA1 as well as for GFP-PCNA1 in PCNA1-GFP over-expressing parasites but not in PCNA2-GFP over-expressing parasites. Similarly, PCNA2 polyclonal immune sera recognized a specific band for endogenous PCNA2 as well as for GFP-PCNA2 in PCNA2-GFP over-expressing parasites, but not in PCNA1-GFP over-expressing parasites. The over-expression of the GFP tagged proteins was further confirmed using commercially available antibodies against GFP. These results confirm the authenticity of the antibodies raised against PfPCNA1 and PfPCNA2 and rules out the possibility of cross reaction.

**Expression and Subcellular localization of PfPCNAs in different intra-erythrocytic stages of the parasite life cycle**

Expression profile of both the proteins was checked by western blot analysis of different blood stages parasites using antibodies against PCNA1 and PCNA2. The results showed that both PfPCNA1 and PfPCNA2 proteins are expressed throughout the ring (16±2 hrs), trophozoite (34±2 hrs) and schizont (42±2 hrs) stages (Figure 1.4 A). However, the expression during Ring stage for both the proteins is very low. PfPCNA1 expression peaks during the replicating trophozoite stage and further

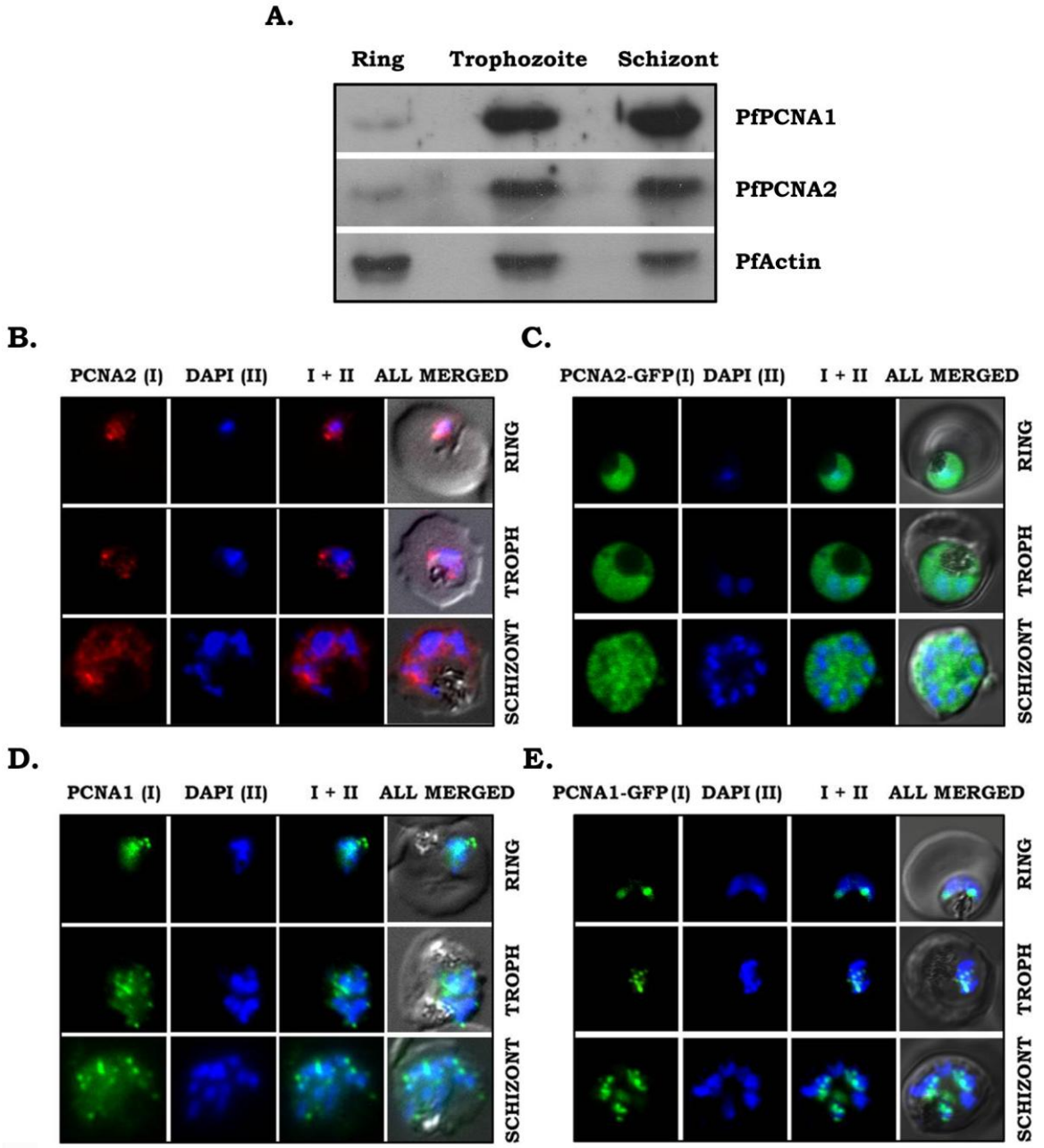


**Figure 1.4. Both PfPCNAs express during blood stages of the parasite. (A) WB analysis of different blood stages of parasite using anti-PCNA1 and PCNA2 specific antibodies.** PfPCNA1 expression is very low during ring stage and it increases significantly during trophozoite and even further at schizont stages of the parasite while that of PfPCNA2 peaks during trophozoite and persist through the schizont stage. PfActin is used as a loading Control. **(B-E) Sub-cellular localization of PfPCNAs.** IFA was performed on glass slides containing parasite smears from an unsynchronized culture, which were treated with anti-PfPCNA1 or anti-PfPCNA2 antibodies or both. **(B)** PfPCNA2 show both nuclear and cytoplasmic diffused staining in the ring and trophozoite stages and nuclear and perinuclear enrichment becomes visible at the schizont stage of the parasite. **(D)** PfPCNA1 (green) shows foci formation from the ring stage to replicating trophozoite stages and multinucleate schizont stages. Live cell imaging analysis of parasites over-expressing PCNA2-GFP **(C)** and PCNA1-GFP **(E)** gave similar localization pattern.

increases in the schizont stage while PfPCNA2 expression peaks at trophozoites and remain unchanged during schizont stage. Previous reports also suggest for the increased PfPCNA1 protein expression in the replicative trophozoite stage along with other components of the replisome (Li, Warren et al. 2002, Patterson, Whittle et al. 2002).

Further, we performed immuno-fluorescence assay to check the sub cellular localization of endogenous PCNA1 and PCNA2 proteins using respective antibodies. The localization of PCNA1-GFP and PCNA2-GFP was studied using live cell imaging. It has been shown earlier that endogenous PCNA1 as well as PCNA1-GFP show nuclear punctate staining pattern following immuno-fluorescence assay or live cell imaging, respectively. These foci mark the active sites of DNA replication as PCNA is a component of replisome complex (Gupta, Mehra et al. 2008). We were interested in studying sub-cellular localization of PfPCNA2 using immune-fluorescence assay and with the aid of PfPCNA2-GFP over-expressing parasite line. We found that PfPCNA2 forms a diffused distribution pattern within the cytoplasm and around the nucleus across various asexual stages of *P. falciparum.* This is in correlation with diffused pattern of the protein when over-expressed as GFP tagged by live cell imaging analysis (Figure 1.4 B-C).
Endogenous PfPCNA1 and PfPCNA1 when over-expressed as GFP fusion proteins in the parasites formed punctate foci with and surrounding DAPI in all the representative stages of the *P. falciparum* IDC under the same experimental conditions (Figure 1.4 D-E).

**Co-immunofluorescence and Co-immunoprecipitation studies of endogenous PfPCNA proteins**

Next, we were interested to check the possibility of interaction of *P. falciparum* PCNAs between each other. For this purpose, we performed co-localization and co-immunoprecipitation studies for PfPCNA1 and PfPCNA2. We performed co-immunofluorescence assays using antibodies against PfPCNA1 (Rabbit) and PfPCNA1 (Mouse). We observed partial co-localization of these two proteins during different erythrocytic stages (Figure 1.5 A). Subsequently, co-immunoprecipitation experiments were performed where antibodies specific to PfPCNA1 or PfPCNA2 protein was incubated with protein extract prepared from asynchronous 3D7

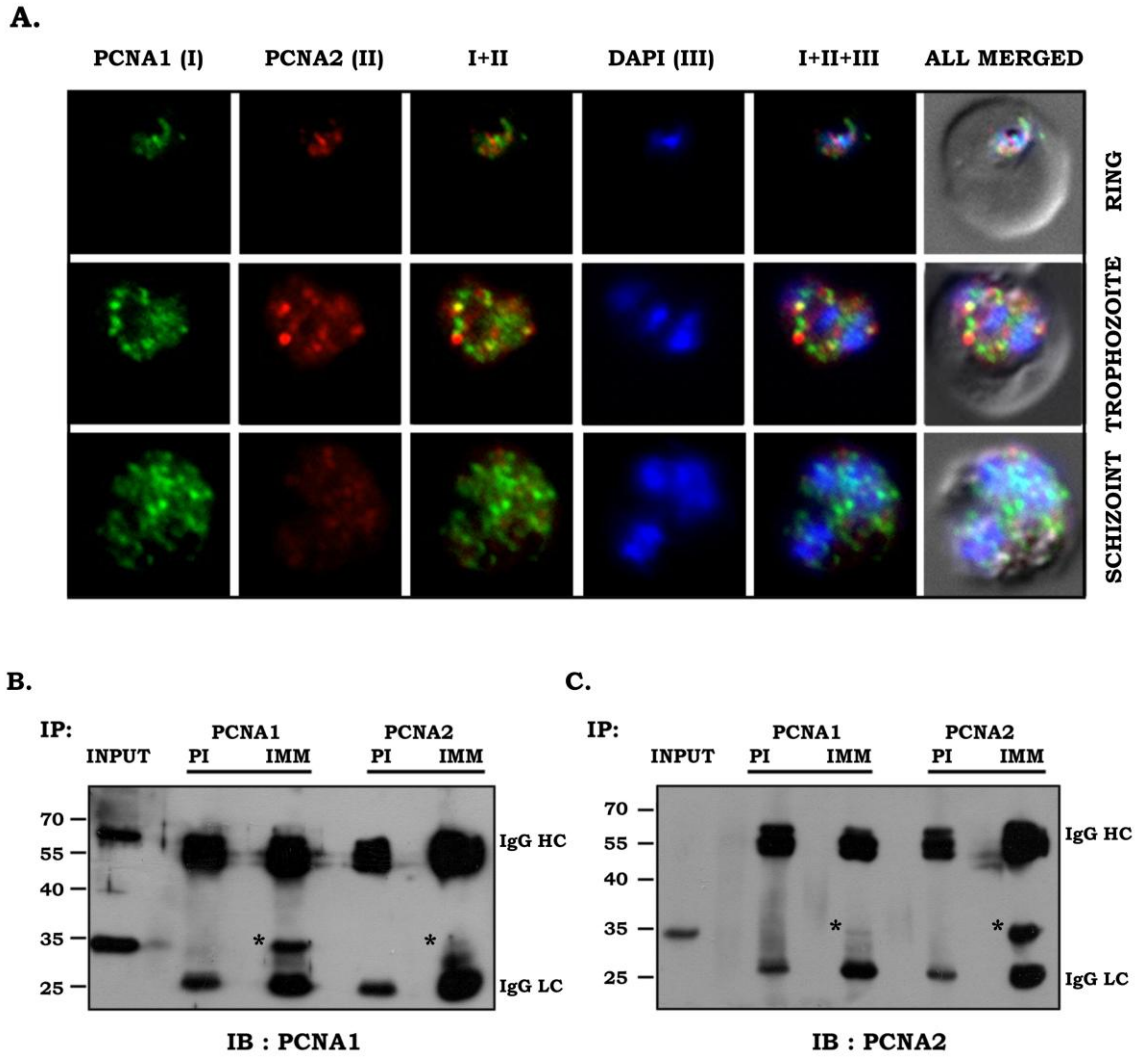


**Figure 1.5. (A) Co-immunolocalization studies between PfPCNAs.** Co-Immunofluorescence assay for PfPCNA1 and PfPCNA2 using specific antibodies raised in different host animals. The results show that the proteins partially colocalize with each other. DAPI (4, 6-diamidino-2-phenylindole) (blue) shows the nuclei. **(B-C) Co-immunoprecipitation assay using parasite lysate to demonstrate interaction between PfPCNA1 and PfPCNA2.** Polyclonal Immune sera against PfPCNA1 or PfPCNA2 were incubated with lysate prepared from *P. falciparum* 3D7 parasites. 5% Parasite extract prepared is taken as input in each case and preimmune sera incubated with parasite extract under similar conditions are taken as the negative control. Antibodies against PfPCNA2 were able to pull down endogenous PPCNA1, confirmed by western blot analysis using antibodies against PfPCNA1 **(B)**. Similarly, antibodies against PfPCNA1 were able to pull down endogenous PPCNA2, confirmed by western blot analysis using antibodies against PfPCNA2 **(C)**. Both the antibodies can pull down the respective endogenous proteins efficiently.

parasites followed by western blot analysis using antibodies against both the proteins. We found that PfPCNA2 was able to pull down endogenous PCNA1 from the lysate prepared from the 3D7 parasites as demonstrated by western blot analysis using antibodies against PfPCNA1 and vice versa (Figure 1.5 B-C). However, the intensity of the pulled down bands were faint suggesting weak interaction between these proteins.

These experiments suggests for the possibility of a heterotrimer complex formation which may be required for DNA metabolism of parasite lifecycle.

## DNA damage and PfPCNAs

Earlier DNA damage studies carried out in our laboratory involving PfPCNA1 have shown that in response to genotoxic agents like MMS (an alkylating agent) and HU (a Ribonucleotide reductase inhibitor), the protein undergoes up-regulation which seems to be regulated at post-transcriptional level. The transcript levels of PfPCNA1 remained constant before and after DNA damage. Episomal expression of PCNA1-GFP under *Pfcrt* promoter also showed similar up-regulation following DNA damage. Although involvement of PfPCNA1 in DNA damage function has been studied but the role of PfPCNA2 in DNA damage response has not been explored yet. Therefore, we were interested to check the behavior of PfPCNA2 in similar conditions of genotoxic stress. For DNA damage studies a DNA damage marker was required. For this purpose, Rad51 in *Plasmodium falciparum* was the most suitable candidate as RecA homologue, PfRad51, has been reported earlier (Bhattacharyya and Kumar 2003) to be involved in HR (homologous recombination) and DNA-repair activities (Baumann and West 1998). It has also been shown to be transcriptionally upregulated in response to DNA damage caused by MMS which induces Single stranded and double stranded breaks.

**Polyclonal antibody generation against the PfRAD51 - DNA damage marker**

Full length $His_6$-PfRad51 protein was Ni-NTA affinity purified from *E. coli* BL21 codon plus cells and injected in rabbit and mice, respectively as per the protocol described in materials and methods section. The full length *Pfrad51* was already cloned in our laboratory by Dr. Pallabi Mitra in pET28a (+) vector. The specificity of the obtained immune sera was checked by Western blotting using uninduced and

IPTG induced bacterial lysate expressing recombinant $His_6$-PfRad51 protein as well as purified recombinant $His_6$-PfRad51 protein. *P. falciparum* 3D7 parasite lysate obtained upon treatment with HU (10mM) and MMS (0.005%) were also used for

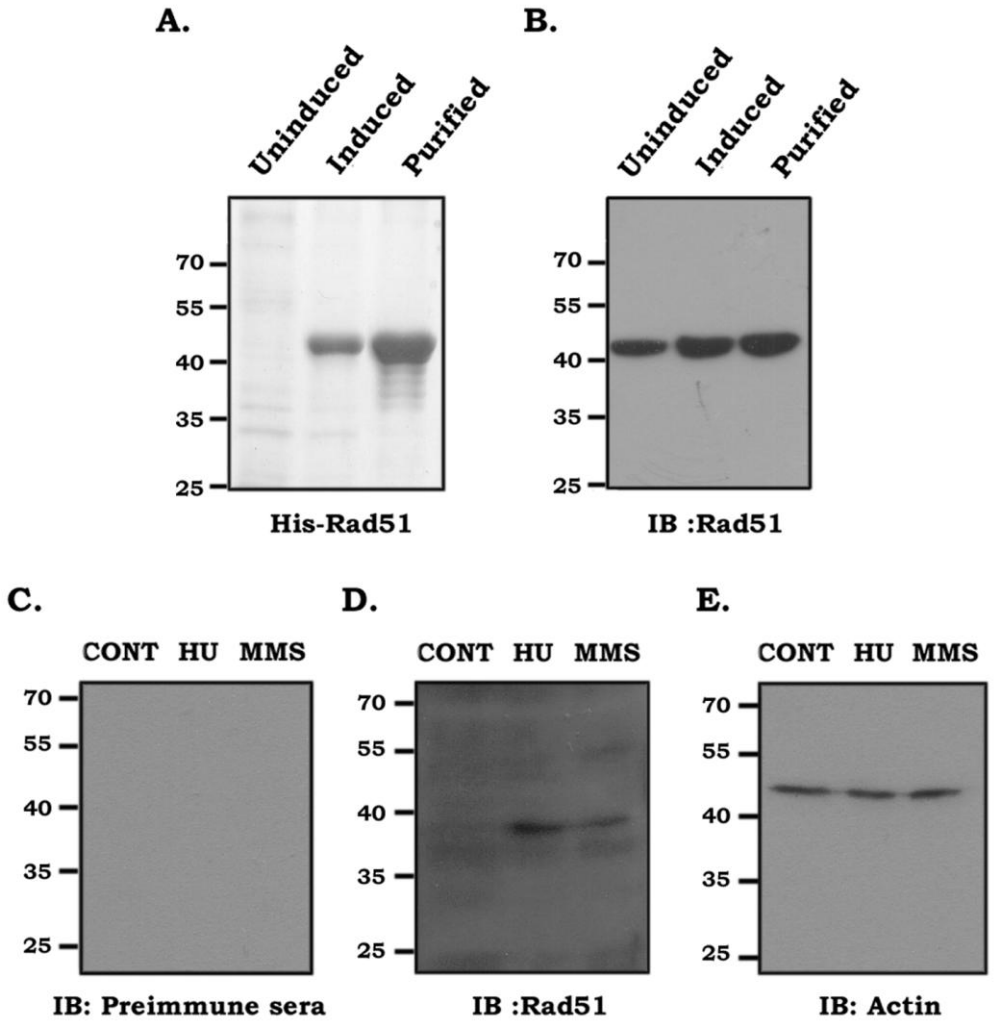


**Figure1.6. Purification profile of His-tagged PfRAD51 protein and Western blotting to show the specificity of PfRAD51 (A-E) antibodies. (A)** Purification profile of $His_6$ PfRAD51. Coomassie stained gel shows the induction of PfRAD51 protein in *E. coli* BL21-DE3 cells as well as the purified protein after Ni-NTA affinity purification. **(B)** Western blot analysis of the proteins as shown in (A) using antibodies against PfRAD51. **(C-D)** The expression of endogenous PfRAD51 was checked by pre-immune sera or antibodies against PfRAD51 in *P. falciparum* lysate treated with HU (10mM) and MMS (0.005%). An expected band at ~38 KDa was observed in HU or MMS treated parasite lanes (since the protein is inducible). **(C)** The corresponding immunoblot with pre-immune sera was clean. **(E)** Western blotting using antibodies against PfActin show equal loading in all the lanes.

Western blot analysis. The antibodies recognized a specific band at ~38 KDa (Figure1.6 B, D). However, no signals were observed when pre-immune sera collected before priming the animals for antibody generation was used (Figure 1.6 C). Immune sera detected a specific band corresponding to $His_6$-PfRad51 in the IPTG induced bacterial lysate as well as in the lane containing purified protein (Figure 1.6 B). A specific band was also observed in parasite lysate following treatment with genotoxic agents (Figure 1.6 D). Pre-immune sera did not recognize any such band under the same experimental conditions (Figure 1.6 C).

**Effect of genotoxic agents on PfPCNAs**

The regulation of PfPCNA2 under genotoxic agents (HU and MMS) was assessed using under similar conditions as those were used for PfPCNA1. Interestingly, we found that PfPCNA2 as well underwent similar upregulation at the protein level in 3D7 strain of *P. falciparum* (Figure 1.5) during late trophozoite stage (~36 h) parasites (n=3, PfPCNA1,P=0.046 for MMS and 0.021 for HU; PfPCNA2,P=0.025 for MMS and 0.045 for HU, respectively). The expression level of PfRAD51 was also upregulated under the same experimental condition in the presence of HU and MMS, respectively confirming that DNA damage indeed took place in the parasites (Figure 1.7 E).

Further, we checked whether the upregulation of PfPCNA1 is specific for any particular stage of development. For this purpose, synchronized *P. falciparum* parasites from mid-trophozoite stage (~26-28 hrs) and late trophozoite stage (~36 hrs) were treated with genotoxic agents as described in materials and methods section followed by western blot analysis of the parasite lysate using antibodies against PfPCNA1 as shown in Figure 1.7 F. The results indicate that the robust upregulation of PfPCNA1 takes place during late trophozoite stage suggesting the importance of the proteins during late stages of the parasite development under genotoxic stress conditions.

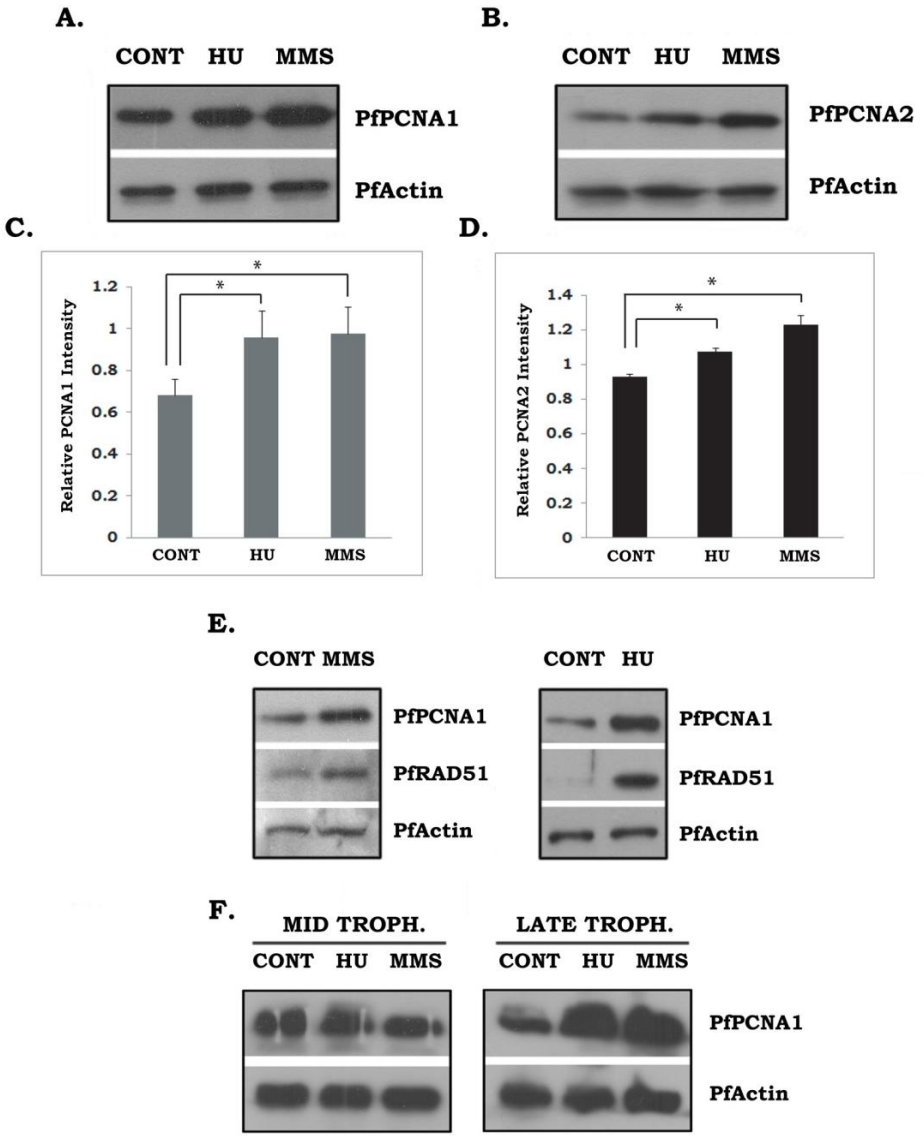


**Figure 1.7. Effect of genotoxic agents on expression of endogenous PCNA1 and PCNA2 proteins**. **(A-B)** Synchronized replicating stage parasites of 3D7 strain were exposed to 10mM HU and 0.005% MMS for 6 hours and expression of both PCNA1 and PCNA2 was checked using WB analysis using antibodies against PCNA1 and PCNA2, respectively. Both showed upregulation of expression at the protein level. **(C-D)** Graphical representation of the quantified signals obtained from densitometric analysis of the intensity of the bands from Western blots and normalized with Actin as mentioned in the materials and methods. The results are mean±S.E.M. from three independent sets of experiments (n=3; PfPCNA1, P=0.046 for MMS and 0.021 for HU; PfPCNA2, P=0.025 for MMS and 0.045 for HU, respectively). (*P<0.05 and **P<0.01) (Student's t test). PfActin is used as the loading control in each case. **(E)** PfRad51 showed upregulation upon DNA damage with MMS and HU under similar conditions. **(F)** Western blot analysis showing stage specific accumulation of PfPCNA1 on induction of DNA damage. PfPCNA1 protein levels show robust increment in the late trophozoite stage (~36 hrs) compared to mid trophozoite stage (~26-28 hrs) parasites upon genotoxic agent (HU and MMS) treatment as determined by with PfPCNA1 specific antibodies. PfActin remains unaltered and is used as a loading control.

**Effect of other DNA damaging agents like Ionizing radiations on PfPCNAs**

We also investigated whether PfPCNAs undergo upregulation at the protein level in response to other DNA damaging conditions including ionizing radiations. Effect of UV irradiation causing DNA lesions (NER pathways) and Gamma irradiation causing Double strand breaks (DSBs) was assessed. DNA lesions consisting mainly of thymine-thymine cyclobutane pyrimidine dimers (CPD) or thymine-cytosine 6-4 photoproducts (6-4 PP) induced by UV light are generally repaired by the nucleotide excision repair pathway (Trotta, Brown et al. 2004). γ irradiation induces double strand breaks in DNA at random sites which are repaired by homologous recombination (HR) or non homologous end joining (NHEJ) mechanism.

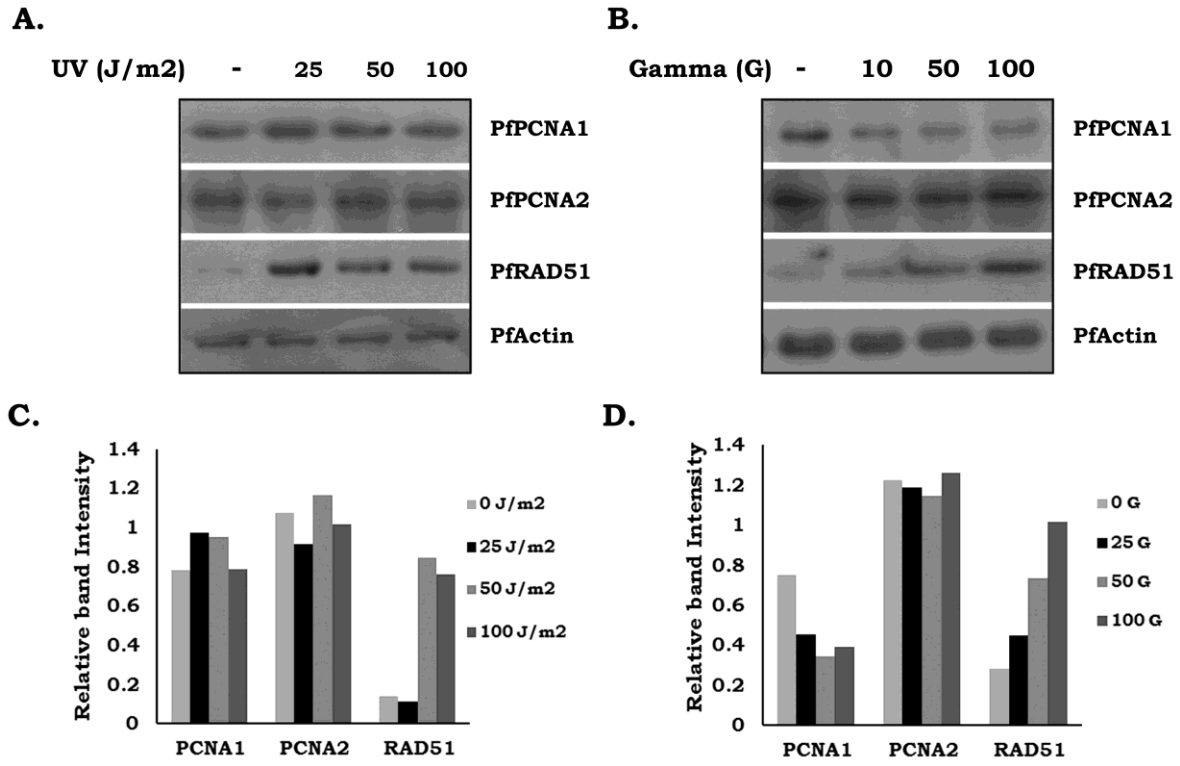


**Figure 1.8. Effect of UV and Gamma irradiation on expression of PfPCNAs**. Synchronized replicating stage parasites of 3D7 strain were exposed to increasing UV dose (25, 50, 100 J/m$^2$) and Gamma dose (10, 50, 100 G) and incubated further for 5 hours and expression of both endogenous PfPCNA1 and PfPCNA2 was checked using WB analysis with anti-PCNA1 and anti-PCNA2 antibodies. No effect was observed on PfPCNAs upon damage with UV **(A)** or Gamma **(B)** irradiation as PfRad51 was upregulated in both the cases. PfActin is used as the loading control in each case. **(C, D)** show densitometric analysis for the corresponding western blots (A & B) representing relative band intensities of PfPCNA1, PfPCNA2 and PfRDA51 normalized against PfActin.

For this purpose, we irradiated trophozoite stage 3D7 parasites (~36 h) to increasing UV rays (25, 50, 100 J/$m^2$) and Gamma rays (10, 50, 100 Gy) and incubated further for 5 hours followed by Western blot analysis to assess the expression of both endogenous PfPCNA1 and PfPCNA2 using antibodies against PCNA1 and PCNA2. No significant effect was observed on PfPCNAs upon damage with UV (Figure 1.8 A, C), however upon Gamma irradiation PfPCNA1 showed down regulation (Figure 1.8 B, D). We also checked for PfRad51 protein levels in the same parasite lysate as a DNA damage marker which was upregulated in both the cases (Figure 1.8 A, B). PfActin is used as the loading control. At present, we do not know why the level of PCNA1 goes down in the presence of γ rays.

## DISCUSSION

Proliferating cell nuclear antigen, (prokaryotic homologue of β clamp - processivity factor for DNA polymerases) is one of the key molecules of DNA replication machinery. It is the molecular coordinator of the core DNA replication machinery, as it forms a homotrimeric ring around the DNA strands and primarily provides processivity to the replicating polymerases. Further, it serves as a docking site for proteins involved in DNA damage repair and response, cell cycle regulation and many other proteins of DNA metabolism (Warbrick 2000, Moldovan, Pfander et al. 2007).

In this study, we have successfully generated antibodies against both the PfPCNAs and an important DNA damage response protein PfRad51 which was proved to be a good tool for the whole study. Comparative stage specific expression study of PfPCNAs across intra-erythrocytic cycle showed low expression of both PfPCNA1 and PfPCNA2 during ring stage and it increases significantly as the parasites progressed to trophozoite stage where DNA synthesis occurs. The expression further increased for PfPCNA1 in schizont stage while that of PfPCNA2 persisted in the schizont stage at a similar level (Patterson, Whittle et al. 2002). This corroborates with the RNA expression profile as well and highlights the requirement of the protein in its DNA replicating stage. The presence of the protein till schizont stage where repair resynthesis and DNA repair occurs indicates their possible requirement in these pathways as well.

Previous immunolocalization studies for endogenous PfPCNA1 and live cell imaging using PfPCNA1-GFP parasite from our lab has demonstrated that it forms distinct replication foci (Gupta, Mehra et al. 2008). Localization studies conducted in this study for PfPCNA2 showed both nuclear and cytoplasmic diffused staining across all the stages.

The presence of two PCNAs in *P. falciparum* also raises the question whether the two are functioning independent of each other or works in association with one another. To check for the possibility of heterotrimer formation *in vivo,* we performed co-localization and co-immunoprecipitation studies for PfPCNA1 and PfPCNA2. The proteins were found to partially colocalize in IFA experiments. Further immunoprecipitation experiments showed weak interaction between these proteins. There is a possibility of a heterotrimer complex formation which may be required for parasite DNA metabolism. It is also possible that heterotrimerisation takes place at a particular developmental stage and it needs to be verified further. A recent study involving PfPCNA1 has shown it to be a response regulator of a sensing system similar to the bacterial two component signal transduction pathway (Wu, Cruz et al. 2016). In this study, they have also reported PfPCNA2 to co-immunoprecipitate with PfPCNA1 which again confirms our finding for heterotrimeric PCNA ring formation in *P. falciparum.* In case of *A. thaliana* (Strzalka and Aggarwal 2013) and in Archaea (Dionne, Nookala et al. 2003), the heterotrimerisation between PCNAs has been shown clearly .

The parasite faces extensive DNA damage from various sources during its life cycle as it encounters oxidative free radicals, free haem, innate immune pressure of the host, errors arising due to a fast rate of DNA replication etc. In order to survive parasites must have evolved with several promising DNA repair mechanisms.

Previous studies from our lab have shown PfPCNA1 to stabilize at protein level in response to DNA damage using MMS and HU. MMS alkylates DNA bases and induces single strand and double strand breaks (Pegg 1984, Lawley 1989), and Hydroxyurea (HU) targets ribonucleotide reductase thereby depletes the dNTPs pool causing a replication fork arrest and subsequent genomic instability (Ahmad, Kirk et al. 1998).

We checked the effect on PfPCNA2 protein under similar DNA damaging conditions. Interestingly, we found that PfPCNA2 as well undergo similar up regulation at the protein level as is the case with PfPCNA1. We also wanted to know whether PFPCNAs upregulate similarly with other DNA damaging agents. We performed DNA damage experiments using UV and γ radiation as well in trophozoite stage (~36 hrs) of 3D7 strain of *P. falciparum.* We did not observe a considerable change in protein levels of PfPCNA1 or PfPCNA2 upon exposure to varying doses of UV or γ rays; however, PfRad51 showed increased protein levels under the same experimental conditions confirming activation of DNA damage response pathways.

PCNA is implicated in repair resynthesis step and repair patches may vary from 5-7 bp in BER (Haltiwanger, Karpinich et al. 2000, Haltiwanger, Matsumoto et al. 2000) and 25-30 bp in nucleotide excision repair (NER) (Gary, Ludwig et al. 1997, Gary, Park et al. 1999), in case of bulky lesions as is the case with UV damage. However, Gamma causes randomly distributed double strand breaks (DSBs) in the DNA which are repaired by Homologous recombination (HR) and Non Homologous End Joining (NHEJ).

In *P. falciparum,* components of BER pathway (Haltiwanger, Matsumoto et al. 2000) along with a putative NER pathway (Trotta, Brown et al. 2004) have been identified. In *P. falciparum* PCNA has been shown to be involved in Long patch Base Excision repair pathway (**LP-BER**) (Haltiwanger, Matsumoto et al. 2000). The presence of a functional *E. coli Rec A* homolog, *Rad51* (Roy, Bhattacharyya et al. , Bhattacharyya and Kumar 2003) along with other HR components Rad54 and RPA1 (Gopalakrishnan and Kumar 2013) to repair double strand DNA breaks have also been reported.

DSBs induced by γ irradiation inhibit replicon initiation but does not affect fork progression (Rowley, Phillips et al. 1999). This may account for down regulation of PfPCNA1 and no effect on PfPCNA2 protein levels upon DNA damage with gamma in our study in *P. falciparum*. Perturbation of replicon initiation which is the early step of DNA replication due to γ irradiation may not lead to the requirement of PCNA for later steps of DNA elongation. Further DNA synthesis may be blocked leading to low levels (due to degradation) or basal levels of PCNA.

Formation of DNA adducts in DNA upon UV exposure are repaired by NER where proteins assemble to recognize, incise, and excise the damaged strand from the genomic DNA (Scharer 2003). Similarly, UV-induced inhibition of DNA synthesis or cell cycle progression was enhanced and accelerated PCNA degradation has been demonstrated (Yu, Cai et al. 2009). Another mechanism for damage induced survival upon UV damage was monoubiquitination of PCNA at K164 for error prone TLS polymerase switch or polyubiquitination for error free DNA synthesis in eukaryotes and sumoylation of PCNA in *S. cerevisiae* K164 and K127 by Siz1 E3 SUMO ligase helps prevent uncontrolled recombination (Papouli, Chen et al. 2005, Pfander, Moldovan et al. 2005).

However, in *P. falciparum* at this stage we may not be able to comment on the role of PfPCNAs upon UV damage as the proteins do not show any change as compared to induction upon treatment with potent chemical agents (HU and MMS).

HU causes dNTP block at new replication forks and MMS methylates the bases in DNA inducing stalled replication forks. Therefore, the DSBs are introduced in newly synthesizing DNA. Stability of PCNA may be an outcome of a slowly moving replication fork and assembly of PCNA rings at damaged sites for allowing repair proteins to assemble at damaged sites. Such kind of damage is repaired by HR and BER pathway. In *P. falciparum* upon genotoxic insults with HU or MMS, PCNA stability may help in damage repair through LP-BER pathway.

Later, we were interested to know how this induction of PfPCNAs upon damage with genotoxic agents (MMS and HU) is regulated. Whether it is controlled transcriptionally or post transcriptionally or some post translational modifications are involved. We further probed into the functional relevance of the upregulated PfPCNAs in our next objective.

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# Functional relevance of the Proliferating cell nuclear antigens (PCNAs) of *P. falciparum* in DNA damage response

Previous studies from our laboratory involving PfPCNAs have shown that in response to genotoxic agents, like MMS and HU, the proteins undergoes up-regulation. Moreover, episomally expressed PCNA1-GFP and PCNA2-GFP under *Pfcrt* promoter, (*P. falciparum* chloroquine resistance transporter promoter) showed similar up-regulation as endogenous PfPCNAs following DNA damage suggesting the response to be post-translationally regulated. Indeed qPCR analysis did not show any change in the *pcna1* transcript following DNA damage (Mitra, Banu et al. 2015). It is still not known whether the up-regulation of PfPCNA2 protein following DNA damage is correlated with transcript level. Further we reasoned that over-expression of PfPCNAs in parasites may show some functional relevance with respect to DNA replication and tolerance to DNA damage. It will also be interesting to see whether any post-translational modification of PfPCNAs will render them to become more stable following DNA damage which will justify its up-regulation. This chapter will deal with some of the aspects as described above.

## RESULTS

### Effect of genotoxic agents on expression of PfPCNA2 at transcript level

To investigate whether the increased cellular levels of PfPCNA2 protein is correlated with increased transcript level, we sought to check the mRNA levels of *Pfpcna2* gene upon DNA damage. In 2003, Bhattacharyya and Kumar have documented that Rad51 plays a central role in the repair of double strand DNA breaks mediated via homologous recombination mechanism in *P. falciparum*. They also demonstrated that the expression of *Pfrad51* gene is up-regulated in response to treatment with MMS similar to that observed in a variety of eukaryotic cells (Jang, Jin et al. 1994, Campbell and Romero 1998, Chen, Yuan et al. 1999).

Therefore, we were interested to look at the expression of DNA damage inducible gene, *Pfrad51* in addition to *Pfpcna1* and *Pfpcna2* genes in the parasites at the transcript level in the presence of DNA damaging agent HU. For this purpose, we performed semi quantitative RT PCR analysis from cDNA obtained from total RNA isolated from parasites exposed to HU under similar conditions of DNA damage as described in materials and methods section and observed that *pcna2* transcript level did not changed significantly following DNA damage similar to that of *pcna1* (Figure 2.1 A-

B). However, the transcript levels of *Pfrad51* increased significantly following HU treatment (Figure 2.1 A-B). PfGAPDH has been used as an internal control. These experiments were repeated few times and similar results were obtained. Therefore, it can be concluded that the stability of the proteins are at the post translational level and are not regulated at the transcription level.

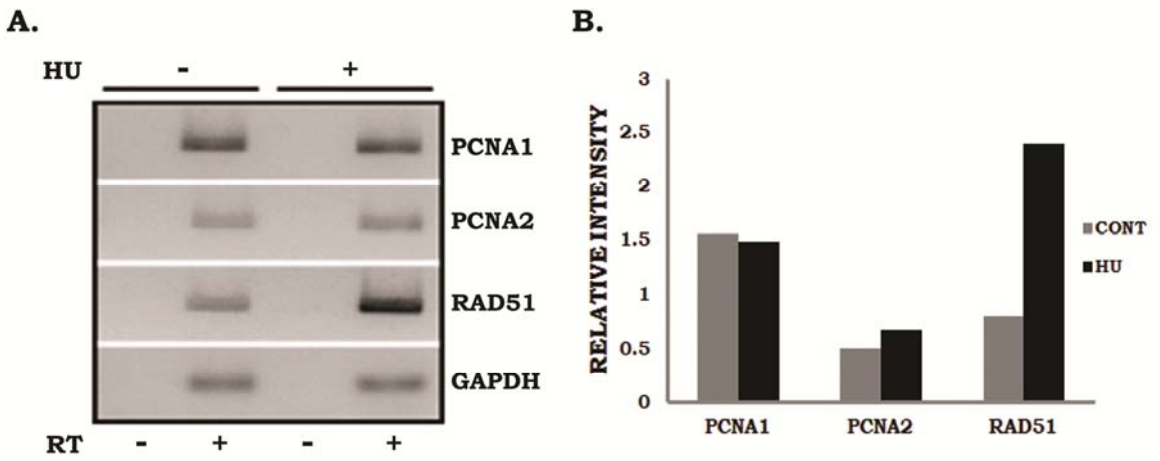



**Figure 2.1. Semi quantitative RT PCR analysis of PfPCNA2 in response to genotoxic stress. (A)** cDNA synthesized from total RNA isolated from untreated and HU treated 3D7 parasites was subjected to RT PCR analysis using gene specific primers to PfPCNA1, PfPCNA2, PfRAD51 and PfGAPDH. PfGAPDH is used as an endogenous control. The absence and presence of reverse transcriptase enzyme while preparing cDNA, is represented as RT – and +, respectively. **(B)** The graphical depiction of the semi quantitative RT-PCR. There is significant up-regulation in PfRad51 transcript on exposure to HU relative to the untreated control.

**PfPCNAs show chromatin enrichment in response to DNA damage**

We wanted to know the functional relevance of increased PfPCNA protein levels in the parasite in response to the genotoxic stress. Therefore, we questioned whether total pool of PfPCNAs is stabilized or proteins are enriched in particular sub-cellular compartments of the parasite when treated with DNA damaging agents MMS and HU. Two populations of PCNA protein are present within a cell: the detergent-resistant form which is chromatin-bound (detergent-insoluble, IN), and involved in DNA replication and repair; and the chromatin-unbound form, that can be readily released by detergent extraction (detergent-soluble, SL) which is not involved in DNA synthesis (Bravo and Macdonald-Bravo 1987). For this purpose, we performed sub-cellular fractionation experiments and isolated the detergent soluble (SL) and

insoluble (nuclear) fractions (IN) of the untreated and drug treated parasites as described in the materials and methods section. These fractions were then electrophoresed and subjected to western blot analysis using antibodies against PfPCNA1 and PfPCNA2.

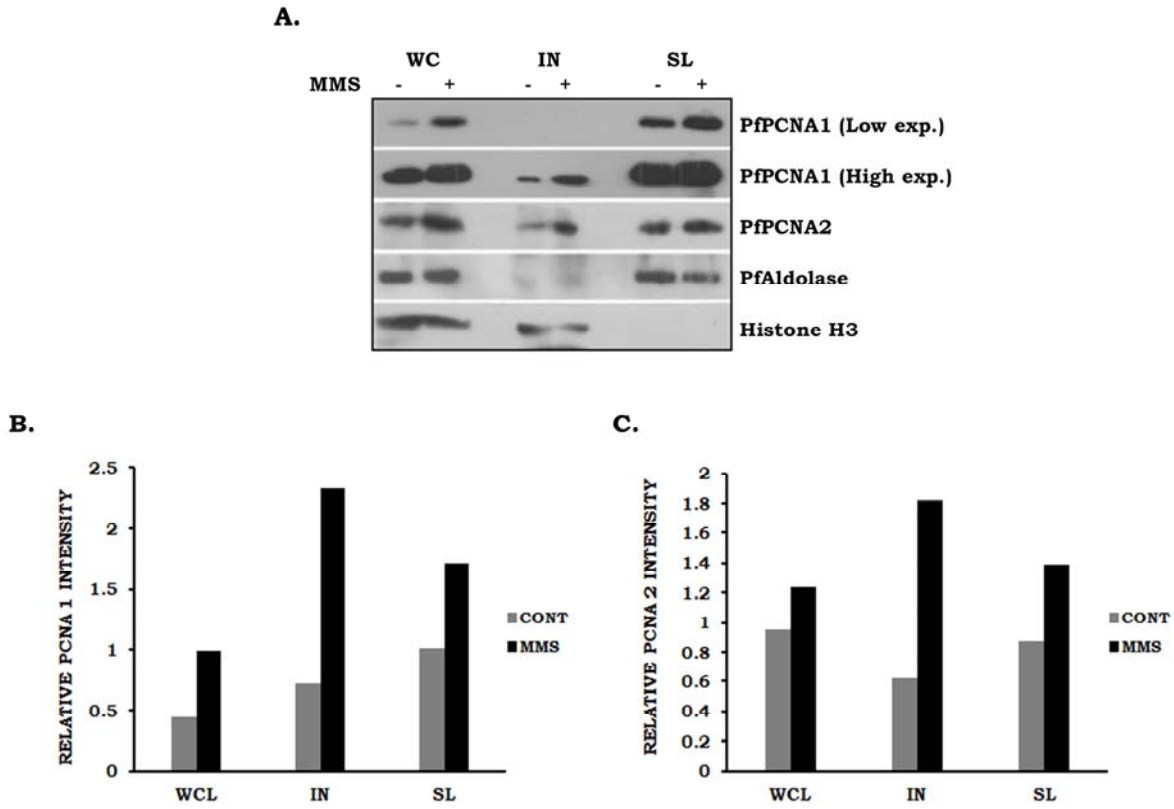



**Figure 2.2. Chromatin enrichment of PfPCNA proteins upon DNA damage using MMS. (A)** Detergent insoluble (IN, Nuclear) and detergent soluble (SL, Cytoplasmic) fractions were isolated from untreated and genotoxic agent (MMS) treated parasites as described in materials and methods. Western blotting with antibodies against PfPCNA1 and PfPCNA2 showed increment of the proteins in presence of genotoxic agent in both the fractions. Histone H3 is used as a marker for chromatin fraction and PfAldolase is used as a marker for cytoplasmic fraction as well as loading control. **(B, C)** Figures show densitometric analysis of western blots using Image J software for the increment in protein levels of PfPCNA1 (B) and PfPCNA2 (C) in all the sub-cellular fractions of parasites upon MMS treatment. WCL – whole cell parasite lysate, IN – detergent insoluble fraction and SL – detergent soluble fraction.

We found that both detergent soluble as well as insoluble (nuclear) pools of PCNA1 and PCNA2 increased following DNA damage with both MMS (Figure 2.2 A-C) and HU (Figure 2.3). Histone H3 was used as a marker for nuclear fraction. Similarly, PfAldolase (used as a marker for cytoplasmic fraction) could be seen in the cytoplasmic fraction only. The overall upregulation of both PfPCNA1 and PfPCNA2

and subsequent enrichment onto the chromatin clearly suggest the role of these proteins following DNA damage.

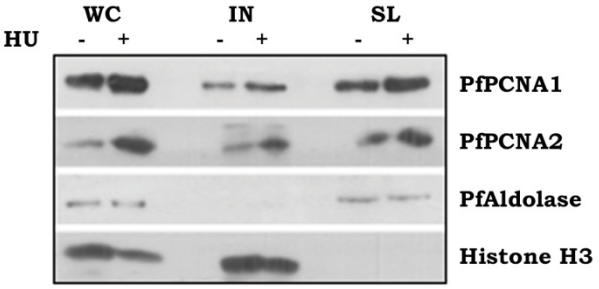



**Figure 2.3. Chromatin enrichment of PfPCNA1 and PfPCNA2 upon DNA damage with HU. (A)** Detergent insoluble (IN, Nuclear) and detergent soluble (SL, Cytoplasmic) fractions were isolated from untreated and genotoxic agent (HU) treated parasites as described in materials and methods. Western blotting with antibodies against PfPCNA1 and PfPCNA2 showed increment of the proteins in presence of genotoxic agent in both the fractions. Histone H3 is used as a marker for chromatin fraction and PfAldolase is used as a marker for cytoplasmic fraction as well as loading control.

**DNA damage response is coincided with increased number of PfPCNA1 foci and nuclear enrichment of PfPCNA2**

Further, we were interested to follow the fate of increased level of PfPCNAs following DNA damage in live cells. Therefore, with the aid of the parasites over-expressing PfPCNA1-GFP and PfPCNA2-GFP, we performed live cell imaging analysis following DNA damage using HU. Analyzing PCNA1-GFP foci post DNA damage (HU), we found that the average number of foci per parasite as well as the intensity of the foci increased considerably when compared to the untreated parasites ($P = 0.0103$) (Figure 2.3 A-B). More than 100 parasites, spanning three separate experiments were analyzed for this purpose. Increased foci formation following the treatment with DNA damaging agents reflects the possibility of the presence of PfPCNA1 at the damaged DNA sites, possibly playing a role in repair resynthesis. With respect to parasites over-expressing PfPCNA2-GFP, distinct nuclear enrichment was seen in HU treated parasites compared to that of untreated parasites (but without the punctate pattern). Quantitative analysis using confocal microscopy showed significant nuclear enrichment of PCNA2-GFP signal following DNA dama-

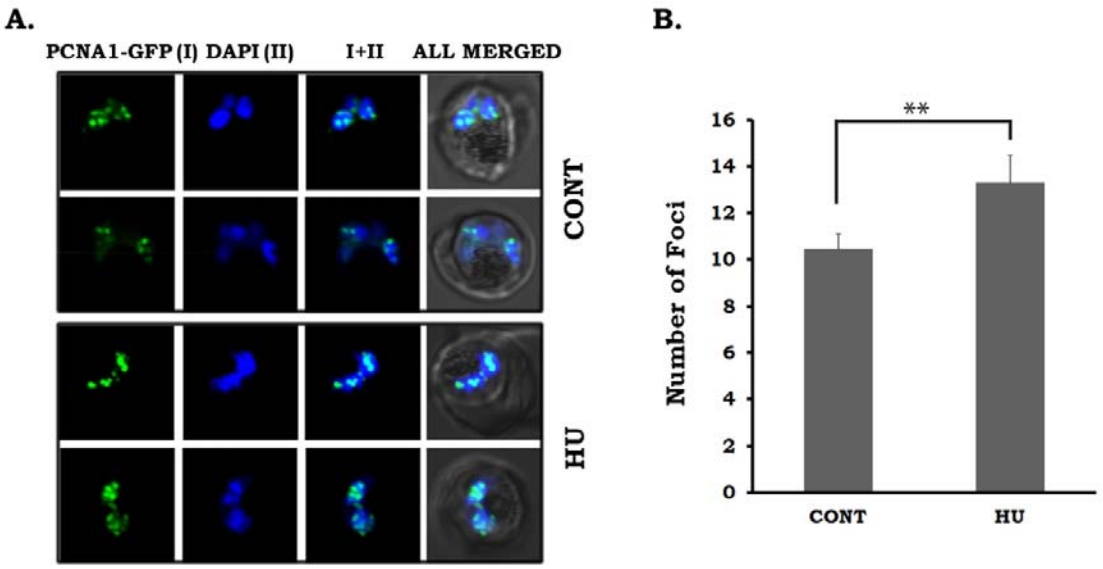



**Figure 2.4. Effect of genotoxic agents on the expression of PCNA1-GFP by Live cell imaging**. **(A)** Representative images of PCNA1-GFP transgenic parasites with nuclear punctuate foci in the absence and presence of genotoxic agent (HU) are shown. (**B**) Average number of foci associated with the nucleus were counted before and after HU damage in more than hundred parasitized erythrocytes from three independent experiments (n=3, P = 0.01) were analysed. (**P < 0.01) (Student's t test). The images were captured and processed using Olympus confocal microscope and Olympus Fluoview FV10-ASW 4.0software.

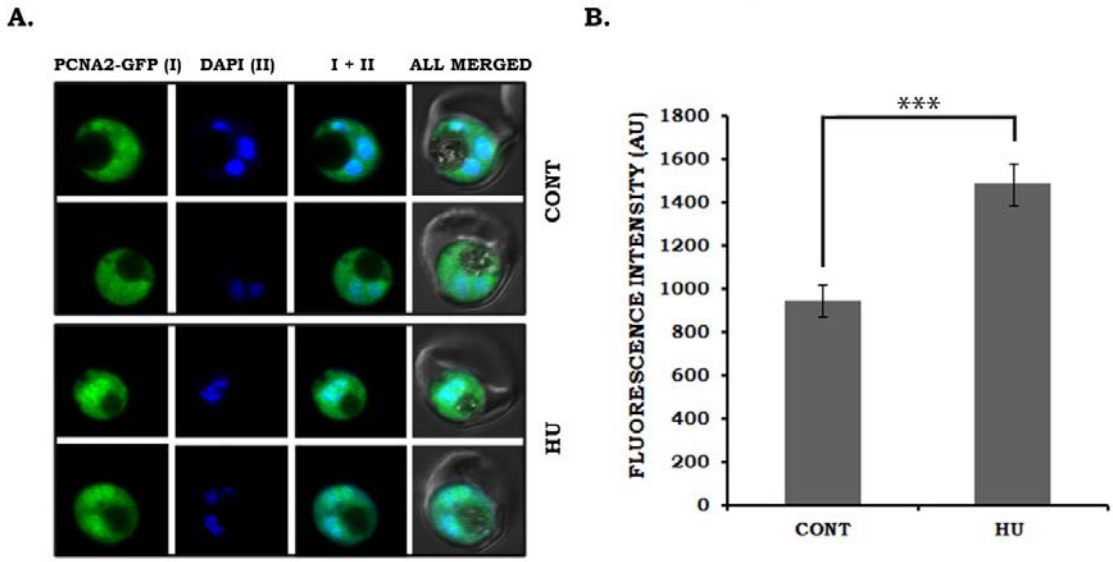



**Figure 2.5. Effect of genotoxic agents on the expression of PCNA2-GFP by Live cell imaging**. **(A)** Representative images of the live cell PCNA2-GFP transgenic parasites in the absence and presence of genotoxic agent (HU) are shown. (**B**) Fluorescence intensity of GFP signals associated with the nucleus was analyzed before and after HU damage in more than hundred parasitized erythrocytes from three independent experiments (n=3, P = 0.0002) and shown graphically (***P <

0.001) (Student's t test). The images were captured and processed using Olympus confocal microscope and Olympus Fluoview FV10-ASW 4.0 software.

-ge (P = 0.0002, Figure 2.4 A-B) suggesting the role of PfPCNA2 in DNA damage response pathway as well.

**Over-expression of PfPCNAs in the parasites confer a survival advantage in response to DNA damage**

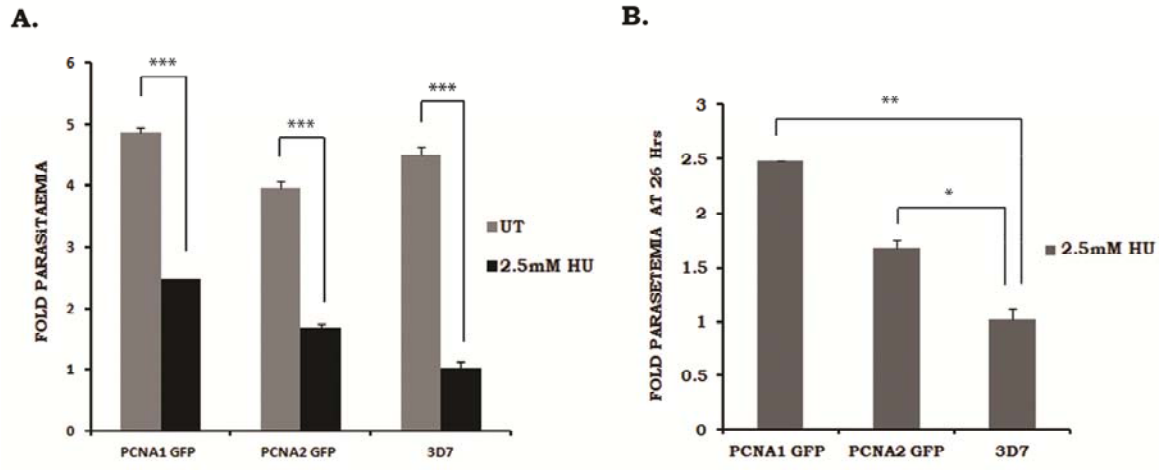
The up-regulation of PfPCNA proteins following DNA damage raises the issue whether over-expression of PfPCNA proteins would have survival edge under the influence of genotoxic agents. Accordingly, we performed *in vitro* parasite survival assays to check the effect of over-expression of PfPCNAs in parasites in genotoxic conditions using the PfPCNA-GFP over-expressed parasite lines. Earlier, such kind of experiments has been performed in another protozoan, *Leishmania major* (Nunes, Damasceno et al.).

For this purpose, the 3D7 parasites and parasites over-expressing PfPCNAs as GFP fusion proteins were treated with the genotoxic agents HU and MMS (at a concentration of 2.5mM HU and 0.002% MMS, respectively) and incubated for 6 hours. The parasites were then washed and allowed to grow in fresh medium for the next cycle. Giemsa staining of the parasites recovered at different time points was performed for the untreated and drug treated parasites as described in the materials and methods section. Interestingly, HU and MMS registered differential survival in untransfected 3D7 and PfPCNA-GFP expressing parasite lines. We found that both PCNA1-GFP (P = 0.0008, 0.001 for HU and MMS, respectively) as well PCNA2-GFP lines (P = $7.5*10^{-5}$, 0.005 for HU and MMS, respectively) could rescue from the damaging effects of the drugs and proliferated well in the next cycle by forming new rings as compared to untransfected 3D7 (Figure 2.6 A-C, 2.7 A-C). The 3D7 strain of *P. falciparum* parasites were mostly arrested in the schizont stage under similar experimental conditions.

Interestingly, the PfPCNA1-GFP over-expression rescued the parasites to a greater extent than PfPCNA2-GFP over-expression in the presence of either HU or MMS (P = 0.002, HU; P = 0.009, MMS for PCNA1-GFP line and P = 0.031, HU; P = 0.024, MMS for PCNA2-GFP line) (Figure 2.6 B, 2.7 B).

To rule out the possibility that the survival edge is not due to the over-expression of any GFP-fusion protein under drug selection pressure, we used an unrelated Sir2-GFP expressing parasite line for the same rescue experiments. PfSIR2 is a histone deacetylase which has a role in the regulation of virulence gene expression, but not in DNA damage response. These parasites did not show considerable survival edge compared to the 3D7 parasites (Figure. 2.8 A-C).

These results suggest that both PCNA1 and PCNA2 play a major role in DNA damage response pathways and subsequent recovery in *P. falciparum* when over-expressed under genotoxic stress condition.

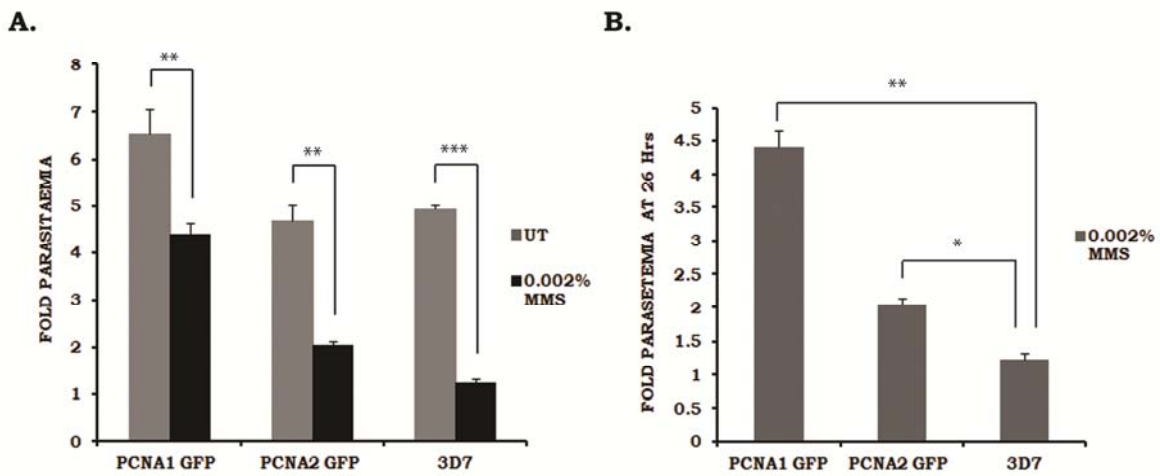



C.

| | PCNA1 GFP | PCNA2 GFP | 3D7 |
|---|---|---|---|
| UT (0 hrs) | 1 | 1 | 1 |
| UT (26Hrs) | 4.87 | 3.98 | 4.5 |
| 2.5 mM HU (26 Hrs) | 2.48 | 1.68 | 1.02 |

**Figure 2.6. Over-expression of PfPCNA1 and PfPCNA2 in the parasites confer survival advantage in the presence of DNA damage by HU. (A)** Synchronized replicating stage *P. falciparum* 3D7, PCNA1-GFP, and PCNA2-GFP expressing parasites maintained at 1% parasitemia were treated with 2.5mM HU for 6 hours. At the end of the treatment, parasites were washed once with complete RPMI media and resuspended in fresh complete RPMI media and followed for different time intervals. At the end of each time point, Giemsa stained slides were prepared and parasitemia was counted. At least 700-800 cells were counted for each treatment and each experiment was done in triplicates. Graphs were plotted for fold increase in the parasitemia for each under control vs. HU treated condition. Error bars represent SEM of three independent experiments. * Represents statistical significance for HU treated PCNA1GFP ($P = 0.0008$), PCNA2GFP ($P = 7.2*10^{-5}$) and 3D7 ($P = 8.6*10^{-5}$) compared with respective untreated counterparts. (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) (Student's t test). **(B)** Graphs showing differential rescue patterns by parasites over-expressing PCNA1-GFP and PCNA2-GFP after HU treatment with respect to

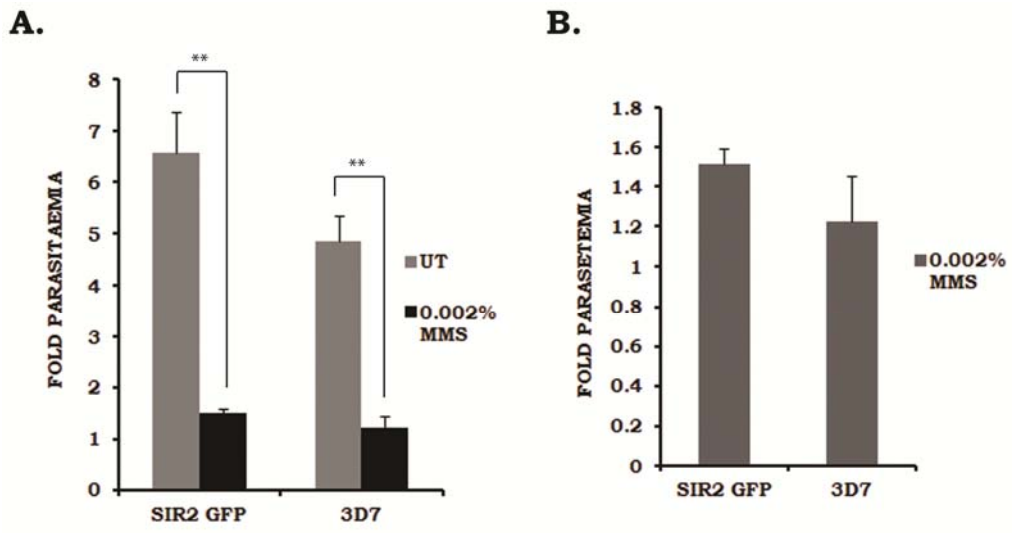
untransfected 3D7 parasites. Error bars represent SEM of three independent experiments. * Represents statistical significance for HU treated PCNA1-GFP vs. 3D7 (P = 0.002) and PCNA2-GFP vs. 3D7 (P = 0.031). (*P < 0.05, **P < 0.01 and ***P < 0.001) (Student's t test). **(C)** Mean values of the fold change in parasitemia for untreated and HU treated parasites at different time points are shown in the table.




C.

| | PCNA1 GFP | PCNA2 GFP | 3D7 |
|---|---|---|---|
| UT (0 hrs) | 1 | 1 | 1 |
| UT (26Hrs) | 6.54 | 4.7 | 4.94 |
| 0.002% MMS (26 Hrs) | 4.4 | 2.05 | 1.3 |

**Figure 2.7. Over-expression of PfPCNA1 and PfPCNA2 in the parasites confer survival advantage in the presence of DNA damage by MMS. (A)** Synchronized replicating stage *P. falciparum* 3D7, PCNA1-GFP, and PCNA2-GFP expressing parasites maintained at 1% parasitemia were treated with 0.002% MMS for 6 hours. At the end of the treatment, parasites were washed once with complete RPMI media and resuspended in fresh complete RPMI media and followed for different time intervals. At the end of each time point, Giemsa stained slides were prepared and parasitemia was counted. At least 700-800 cells were counted for each treatment and each experiment was done in triplicates. Graphs were plotted for fold increase in the parasitemia for each under control vs. MMS treated condition. Error bars represent SEM of three independent experiments. * represents statistical significance for MMS treated PCNA1GFP (P = 0.001), PCNA2GFP (P = 0.005) and 3D7 (P = 0.0001) compared with respective untreated counterparts. (*P < 0.05, **P < 0.01 and ***P < 0.001) (Student's t test). **(B)** Graphs showing differential rescue patterns by parasites over-expressing PCNA1-GFP and PCNA2-GFP after MMS treatment with respect to untransfected 3D7 parasites. Error bars represent SEM of three independent experiments. * represents statistical significance for MMS treated PCNA1-GFP vs. 3D7 (P = 0.009) and PCNA2-GFP vs. 3D7 (P = 0.024). (*P < 0.05, **P < 0.01 and ***P < 0.001) (Student's t test). **(C)** Mean values of the fold change in parasitemia for untreated and MMS treated parasites at different time points are shown in the table.




C.

| | SIR 2 GFP | 3D7 |
|---|---|---|
| UT (0 hrs) | 1 | 1 |
| UT (26Hrs) | 6.59 | 4.87 |
| 0.002% MMS (26 Hrs) | 1.51 | 1.22 |

**Figure 2.8. Over-expression of PfSir2 does not confer significant survival advantage to the parasite with respect to untransfected 3D7 parasites**. **(A)** Synchronized replicating stage *P. falciparum* 3D7 and PfSir2-GFP expressing parasites maintained at 1% parasitemia were treated with 0.002% MMS for 6 hours. At the end of the treatment, parasites were washed once with complete RPMI media and resuspended in fresh complete RPMI media and followed for different time intervals. At the end of each time point, Giemsa stained slides were prepared and parasitemia was counted. At least 700-800 cells were counted for each treatment and each experiment was done in triplicates. Graphs were plotted for fold increase in the parasitemia for each under control vs. MMS treated condition. Error bars represent SEM of three independent experiments. * represents statistical significance for MMS treated PfSir2-GFP expressing parasites vs. 3D7 ($P = 0.008$, Sir2-GFP; $P = 0.018$, 3D7). **(B)** The results indicate that the PfSir2-GFP expressing parasites do not show considerable survival edge compared to the 3D7 parasites under genotoxic environment (**$P < 0.01$) (Student's t test). **(C)** Mean fold change in parasitemia at different time points are indicated in the table.

### Identification of other DNA damage inducible proteins

DNA damage response pathways are crucial for maintaining genomic stability and require assembly of various DNA repair proteins. We were interested to find out DNA damage response proteins apart from PCNAs in *P. falciparum.* For this purpose, we performed 2D gel electrophoresis using parasite extracts from HU

treated/untreated trophozoite stage (~36 hrs) 3D7 *P. falciparum* parasites. Briefly, both HU treated and untreated parasites were lysed and proteins were extracted and quantitated. The protein extracts were then precipitated (Methanol precipitation) to remove excess salts and resuspended in rehydration buffer. Samples were applied to an IPG strip of 13 cm length and pH range 3-10 overnight at 20°C over a uniform horizontal surface. Isoelectric focusing was carried out at 20°C achieving 60,000 volts at the end. IPG strips after isoelectric focusing strips were then equilibrated to reduce and alkylate the resolved proteins and 2$^{nd}$ dimension was performed in a 12% SDS PAGE at 150 volts.

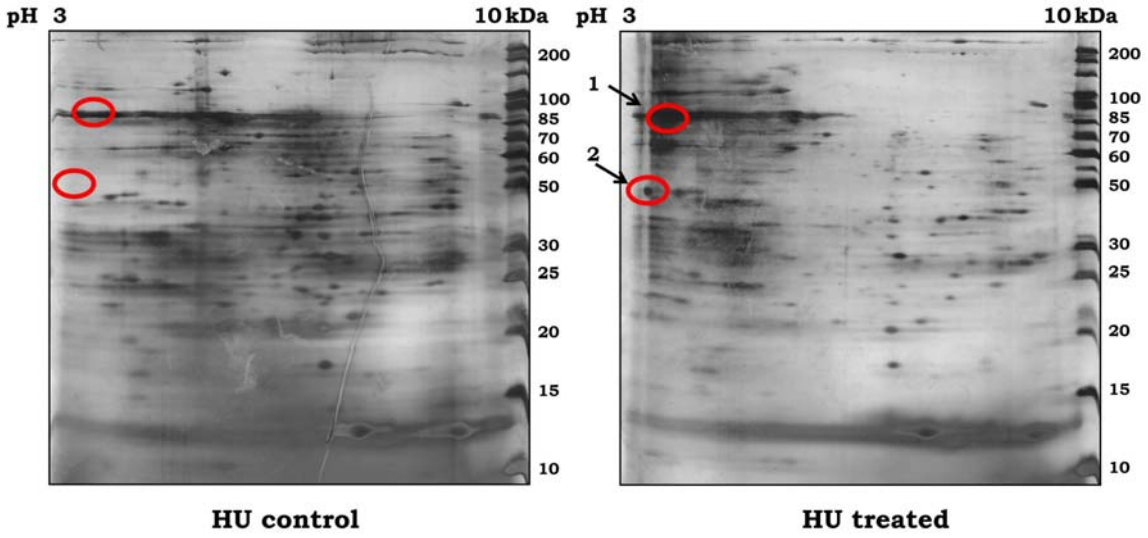



**Figure 2.9. Analysis of proteins following 2DGE in control vs. HU treated parasites. (A)** Proteins extracted from both the HU treated and untreated 3D7 parasites were separated by two-dimensional PAGE (first dimension, pH 3.0–10.0, 13-cm IPG strip; second dimension, 12%), followed by silver staining. Arrows denote the upregulated protein spot (1 and 2) positions.

**Table 2.1: Details of proteins identified from Figure 2.9**

| Spot | Accession | Description | Score | Coverage | Unique Peptides | AAs | MW [kDa] | Cal. pI |
|---|---|---|---|---|---|---|---|---|
| 1 | PF3D7_1329300 | Chromatin assembly factor 1 subunit, putative | 14.86 | 9.97 | 4 | 582 | 66.5 | 5.58 |
| 2 | PF3D7_1224500 | Histone chaperone ASF1, putative (ASF1) | 1.97 | 2.93 | 1 | 273 | 31.5 | 4.49 |

Following 2DGE, gels were silver stained and analyzed for the changes in protein expression in response to DNA damage. We found several protein spots to

upregulate and down regulate following DNA damage as shown in Figure 2.9. We excised few spots and MALDI-TOF analysis was carried out for protein identification. Upon MS analysis we identified proteins from two spots to be upregulated and relevant to DNA damage pathways, i.e. CAF1 and ASF1 (Table 2.1).

We have PfCAF1 specific antibodies in our laboratory generated by Mr. Mohit Gupta (Thesis, Gupta. M, Dhar Laboratory). Therefore, we validated our data by Western blot analysis using 3D7 HU treated parasites under similar experimental conditions. We found CAF1 to upregulate similar to PCNA1 in HU treated parasites, while PfActin remained unchanged (Figure 2.10).

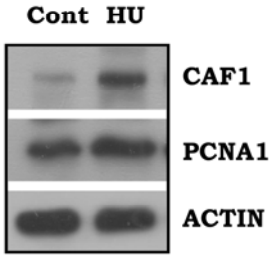



**Figure 2.10 CAF1 upregulates similar to PfPCNA1 upon DNA damage.** Synchronized replicating stage parasites of 3D7 strain were exposed to 10mM HU for 6 hours and expression of both CAF1 and PCNA1 was checked using WB analysis using antibodies against PfCAF1 and PfPCNA1, respectively. Both showed upregulation in expression. PfActin remains unaltered and is used as a loading control.

## DISCUSSION

Every cellular organism encounters different stresses. Similarly *P. falciparum* parasites face the challenging environment within its host and have evolved with various survival mechanisms. In the present study, we attempted to explore the role of PfPCNAs in the presence of genotoxic agents. By far, we knew PfPCNAs are stabilized following DNA damage with well known genotoxic agents (MMS and HU).

The ability to experimentally modulate expression of genes in the parasite enabled us to investigate the gene regulatory mechanism in the parasite. In fact, our study clearly demonstrates the differential gene modulation to identical stress conditions whereby

genes like *Pfrad51* and *PffenI* are transcriptionally regulated while *Pfpcna* genes appear to be post translationally controlled in a genotoxic environment (Mitra, Banu et al. 2015). Altogether, these data conclude that PfPCNAs upregulation is due to the reduced turnover or stabilization of the existing protein in the parasite.

We find that there is a clear enrichment of PfPCNA1 and PfPCNA2 on the chromatin following DNA damage. The functional transition of PCNA from soluble to insoluble or chromatin bound form following DNA damage is considered to reflect the DNA repair activity mediated by Pol δ or Pol ε (Tsurimoto 1999). Chromatin bound PfPCNA may act in a damage signaling pathway as a docking site for the repair proteins to act on the damaged DNA. PfPCNA1-GFP protein was localized to the parasite nucleus and displayed punctate foci at the replicative trophozoite stage. On inflicting DNA damage, we observed an increase in average number of foci per cell. Interestingly, PfPCNA2-GFP protein also showed nuclear enrichment under the same experimental conditions suggesting their possible role in DNA damage response (Mitra, Banu et al. 2015).

To further examine the role of PfPCNAs in parasite DNA damage response, we investigated the effect of over-expression of PfPCNAs on parasite progression in the presence of genotoxic agents. Our data suggest that over-expression of both PfPCNA1 and PfPCNA2 confers better ability to cope with DNA damage. PfPCNA1 line shows better survival than PfPCNA2 line; however, both appear to have edge over untransfected 3D7 line in terms of improved recovery from DNA damage stress. It still needs to be probed if the survival advantage is due to improved DNA repair.

We also find another *P. falciparum* protein i.e. CAF1 by 2DGE followed by mass spectrometric analysis to upregulate in a manner similar to PfPCNAs. CAF-1 is a classical histone chaperone that binds histones and promotes nucleosome assembly during DNA replication, DNA repair and gene transcription (Groth, Rocha et al. 2007). CAF-1 preferentially deposits histones onto replicating DNA mediated via its interactions with PCNA (Shibahara and Stillman 1999, Moggs, Grandi et al. 2000). Asf1 is also involved in DNA replication-coupled nucleosome assembly apart from its role in transcriptional gene silencing (Mello, Sillje et al. 2002). Asf1 and CAF-1 cooperate to assemble specifically modified histones during DNA synthesis. Thus, it

is possible that the functions of CAF-1 and PCNA in maintenance of genome stability are mediated through their interactions with different partners or pathways. However, in *P. falciparum* it still needs to be explored how CAF1 protein stability is regulated, whether at transcriptional or translational levels? We are in progress to study the same in collaboration with Mr. Mohit Gupta, who is primarily studying PfCAF1 and other CAF complex proteins in our laboratory. It would be interesting to find out the role of CAF proteins in maintaining genomic stability.

This study has contributed to our understanding of PfPCNAs in DNA damage response in addition to DNA replication. We successfully demonstrated the involvement of both the PfPCNAs in the parasite DNA damage response pathway along with PfRad51. Increase in cellular levels of PfPCNA proteins is not accompanied by a simultaneous increment of the transcript level during DNA damage response. PCNA proteins show chromatin enrichment upon DNA damage and over-expression of PfPCNA1 and 2 confers a survival advantage to the parasite in the presence of genotoxic stress.

Identification of PfCAF1 and PfAsf1, apart from PfPCNAs (Mitra, Banu et al. 2015) and RAD and RPA proteins (Gopalakrishnan and Kumar 2013) in *P. falciparum* provides further insight into DNA repair pathways and an interplay between these proteins can be explored further.

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# Implication of phosphorylation of PfPCNAs with respect to DNA replication and damage response

The results described in the previous chapters clearly suggest the importance of PfPCNAs in DNA damage response. However, the mechanism for the stability of PfPCNAs following DNA damage is not yet clear.

We have shown that there is no effect at the transcript level of PfPCNAs following DNA damage suggesting the possibility of a posttranslational regulation leading to the protein stability. Various post translational modifications (PTMs) like phosphorylation, sumoylation and ubiquitination have been reported for PCNA in other systems. The importance of phosphorylation of Y211 of HsPCNA for chromatin loading of PCNA and its role in cell survival has also been shown (Wang, Nakajima et al. 2006). Another study has shown that the UV irradiation of human quiescent fibroblasts leads to three-fold increase in PCNA levels relative to that of unirradiated control cells (Prosperi, Stivala et al. 1993). Moreover, it has also been shown that DNA damage by UV induces chromatin enrichment of mammalian PCNA (Balajee and Geard 2001). However, no link between DNA damage; and phosphorylation and chromatin enrichment of PCNA has been demonstrated so far.

Clustal W alignment of PCNAs including *P. falciparum* (PfPCNA1 and PCNA2) and *Homo sapiens* showed tyrosine (Y211 of HsPCNA) residue to be conserved at 213 and 210 positions of PfPCNA1 and PfPCNA2, respectively. Further, a recent phosphoproteome data from schizont stage parasites indicate the presence of a phosphorylated Ser residue at 191 position of PfPCNA1 but not in PfPCNA2 (Treeck, Sanders et al.).

Moreover, previous studies from our laboratory has demonstrated PfPCNAs to undergo phosphorylation by *in vitro* kinase assays using parasite extract as a source of kinase and recombinant PfPCNAs as substrates (Mitra P, Dhar laboratory, unpublished data). PfPCNA proteins carrying the above mentioned Tyrosine and Serine mutations have demonstrated compromised phosphorylation as compared to the wild type PfPCNA1 and 2 by similar *in vitro* kinase assays.

Altogether, upregulation of PfPCNAs following DNA damage, conservation of Tyr residue in both PfPCNA1 and PfPCNA2 and the presence of a phosphorylated S191 residue in PfPCNA1 gives us an opportunity to study the function and regulation of

these proteins during DNA replication and DNA damage. In order to investigate the relevance of the Y213 and S191 residues of PfPCNAs in response to DNA damage, we generated parasite lines over-expressing mutant forms of PfPCNA1 and PfPCNA2 that can be used as tools for this study. Also, we assessed each of these mutant forms of the proteins for any change in structural integrity as compared to wild type proteins. Finally, we also made an effort to assess few possible kinases for their ability to phosphorylate PfPCNAs. The selection of kinases was based on available reports in other systems where PCNA has been shown to be phosphorylated and depending on the kinases of importance for the replication proteins in *P. falciparum.*

**Cloning, heterologous expression and purification of various point mutant forms of of PfPCNA1 and PfPCNA2**

In order to investigate the importance of tyrosine (Y) and serine (S) residues of PfPCNAs *in-vitro,* we wanted to compare the trimerisation properties and molecular conformation of the mutant proteins with respect to their wild type counterparts. For this purpose, we cloned various mutant forms of PfPCNAs using respective primer sets (Table M3 in Material and Methods section) in pET-21c or pET-28a vectors (following the protocol for site directed mutagenesis) and *E. coli* BL21codon-plus cells were transformed with the mutant clones to get respective purified $His_6$-fusion proteins as described in the materials and methods section. With respect to PfPCNA1, Tyrosine at 213 position was replaced with phenylalanine, depicted as Y213F; while serine at 191 position was replaced with alanine, represented as S191A; and a double mutant with both Y213F and S191A, depicted as DM was also generated. Similarly, conserved tyrosine at 210 position of PfPCNA2 was replaced with phenylalanine and represented as Y210F. It is worth mentioning here that the expression pattern and the recovery of all the mutant proteins were similar to that of wild type $His_6$-tagged PCNA1 and PCNA2 purified proteins (by Ni-NTA affinity purification).

**Trimerisation status of PfPCNAs and their point mutants**

Functional form of PCNA exists as a trimer in solution. It forms a trimeric ring encircling the DNA which is free to slide over the synthesizing strand of DNA during

elongation step of DNA replication. We wanted to assess all the mutant forms of PfPCNA proteins for their native conformation of trimer formation compared to that of wild type PfPCNAs. For this purpose, we carried out gel filtration chromatography to check whether these mutations are associated with any structural alterations in the proteins as described in the materials and methods section.

Briefly, these proteins were subjected to size-exclusion chromatography using a 200-GL/300 column (Amersham Biosciences). Initially the column was calibrated using gel filtration standard marker proteins that include aldolase (158 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (14 kDa). A standard plot was obtained by plotting elution volume of a standard protein (corresponding to peak fraction number) against their respective molecular masses (logarithmic scale). Following calibration, all the proteins (500 µg each) were subjected to chromatographic separation under the similar experimental conditions. Peak fractions were collected (0.5 ml each) and analyzed by SDS-PAGE. Elution volume corresponding to the peak fraction was used to deduce the molecular mass of the oligomers of PfPCNAs and their mutants fromstandard graph (Figure 3.1).

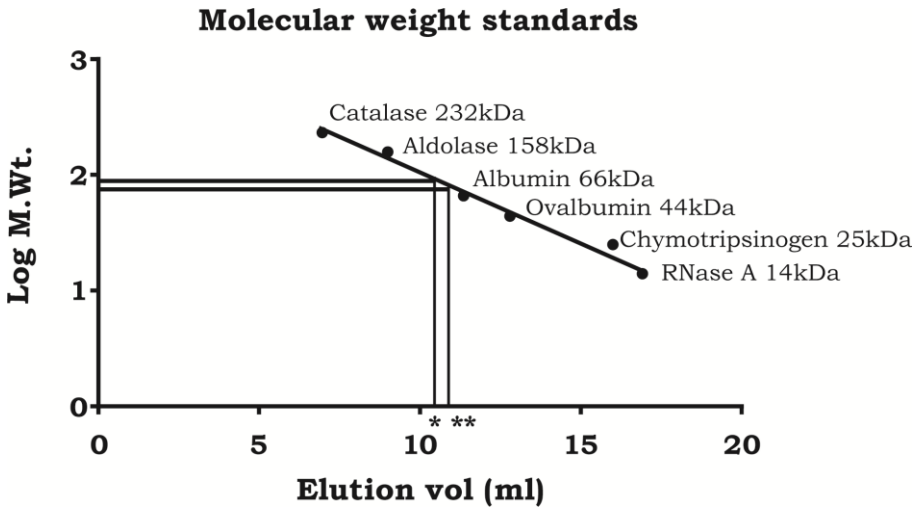



**Figure 3.1. Size-exclusion chromatography of PfPCNAs shows trimer formation in solution.** A standard curve was obtained by plotting the molecular masses of the standard proteins (logarithmic scale) against the peak fraction volume in each case. The peak fraction volume of the wild type PfPCNA1 (*) and PfPCNA2 (**) proteins were plotted in the standard curve and the molecular mass of these proteins were deduced accordingly. The deduced molecular weight for PCNA1 and PCNA2 were 95.4kDa and 91.3 kDa, respectively suggesting trimer forms of these proteins.

Peak fractions for WT-PCNA1 as well as Y213F-PCNA1, S191A-PCNA1 and DM-PCNA1 were eluted at ~10.3 ml elution volume which corresponds to a molecular weight of ~100KDa suggesting the trimer formation in all the cases (Figure 3.2 A-D). Similarly, peak fractions for WT-PCNA2 and Y210F-PCNA2 were obtained at ~10.5 ml

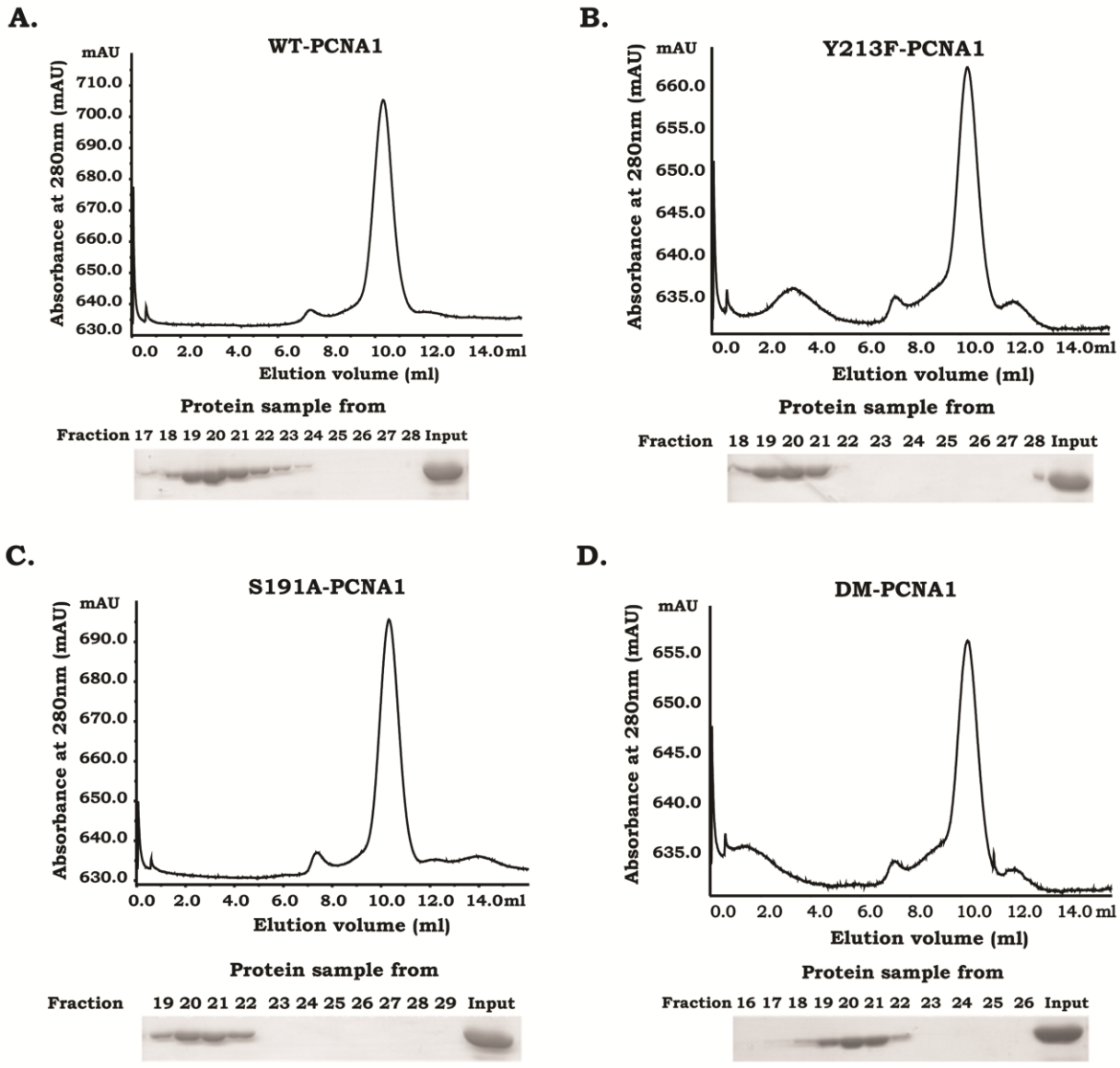



**Figure 3.2. Trimerisation of PfPCNA1 and its mutant forms.** Chromatograms of PfPCNA1 and its mutant forms as obtained by analyzing ~500 µg of each of the WT-PCNA1 **(A)**, Y213F-PCNA1 **(B)**, S191A-PCNA1 **(C)** and DM-PCNA1 **(D)** proteins through 200-GL/300 column (Amersham Biosciences) are shown. WT-PCNA1 as well as mutant forms of PCNA1 were eluted at ~10 ml elution volume suggesting the formation of trimers in solution. Lower panels show the SDS-PAGE analysis of proteins present in the peak fractions for WT-PCNA1 and mutant proteins.

and ~10.34 ml elution volume, respectively which again corresponds to a molecular weight of ~95kDa suggesting the trimer formation in both the cases (Figure 3.3 A-B ). However, peaks corresponding to lower molecular weight protein (at ~12 ml elution volume) and higher molecular weight (at ~7 ml elution volume) were also observed in the case of Y210F PCNA2. The deduced molecular weight for 12 ml peak fraction was ~58 KDa, while there was no protein in the peak fraction corresponding to 7 ml elution volume as observed upon coomassie staining of the gel following SDS PAGE analysis of various peak fractions (Figure 3.3 B). The peak corresponding ~12 ml elution volume may reflect the dimer form of PfPCNA2 or an unstable trimer. However, the coomassie gel showed protein to be present majorly in trimer peak fraction.

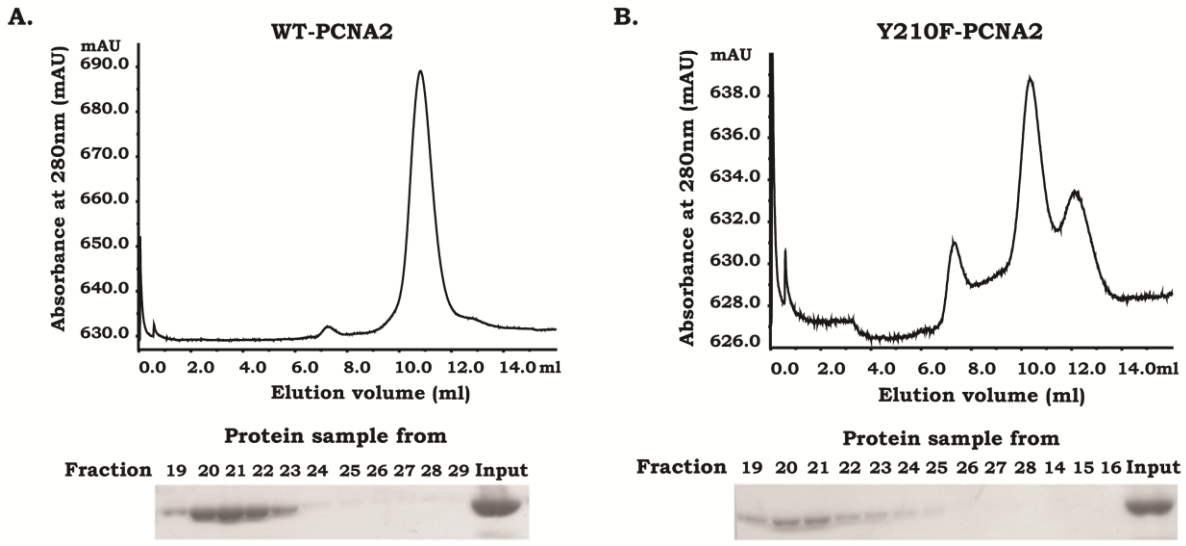



**Figure 3.3. Trimerisation of PfPCNA2 and its mutant Y210F-PCNA2.** Chromatograms of PfPCNA2 and its Y210F mutant as obtained by analyzing ~500 µg of each of the WT-PCNA2 **(A)** and Y210F-PCNA2 **(B)** proteins through 200-GL/300 column (Amersham Biosciences are shown. Both WT-PCNA2 and Y210F-PCNA2 were eluted at ~10 ml elution volume suggesting the formation of trimers in solution, a fraction of Y210F-PCNA2 eluted at ~12 ml volume which corresponds to a dimer, while the peak fraction (14-16) corresponding to ~7 ml volume did not show the presence of protein in coomassie stained gel. Lower panels show the SDS-PAGE analysis of proteins present in the peak fractions for WT-PCNA2 and its Y210F mutant protein.

Altogether, gel filtration chromatography results indicate that all mutants retained their trimerisation properties in solution similar to the wild type PfPCNA proteins. However,

Y210F mutation in PCNA2 may lead to some structural instability that may lead to lower oligomeric forms.

**FAR UV Circular Dichroism (CD) analysis of PfPCNA mutant proteins**

Since, all the mutants were capable of trimerisation similar to their wild type forms; we wanted to compare their molecular conformation with that of wild type protein by far UV Circular Dichroism (CD) analysis. CD is widely used technique in the study of proteins because CD spectra are remarkably sensitive to molecular conformation (Martin and Bayley 2002). Circular dichroism (CD) accounts for the helix content in a polypeptide based on the presence of alpha helix, beta sheet and random coils (Baumruk, Pancoska et al. 1996). Useful information about protein structure, the extent and rate of structural changes and ligand binding can be achieved using CD (Kelly and Price 2000). We performed CD spectroscopy of wild type and mutant PfPCNA proteins as described in the materials and method section.

CD analysis data obtained using Prodata viewer software for PfPCNAs and their point mutant proteins are shown in Figure 3.4 and the α helix and β sheet secondary structure content determination for each protein was done using K2D2 software (Table 3.1).

PfPCNA1 carrying Y213 or S191 mutation did not show considerable upward shift with respect to WT-PCNA1 as observed from CD spectra for each protein (Figure 3.4 A). However, PCNA1 DM showed a greater upward shift relative to the WT-PCNA1 i.e. towards less stable form suggesting loss of stability to some extent upon simultaneous mutation at both Y213 and S191 residues of PfPCNA1. PfPCNA2 carrying Y210 mutation also showed upward shift with respect to WT-PCNA2 as observed from CD spectra (Figure 3.4 B).

These results suggest that some structural changes may have occurred upon simultaneous mutations of both Y213 and S191 residues of PfPCNA1; however individual point mutation does not lead to a drastic change in molecular conformation of the protein.

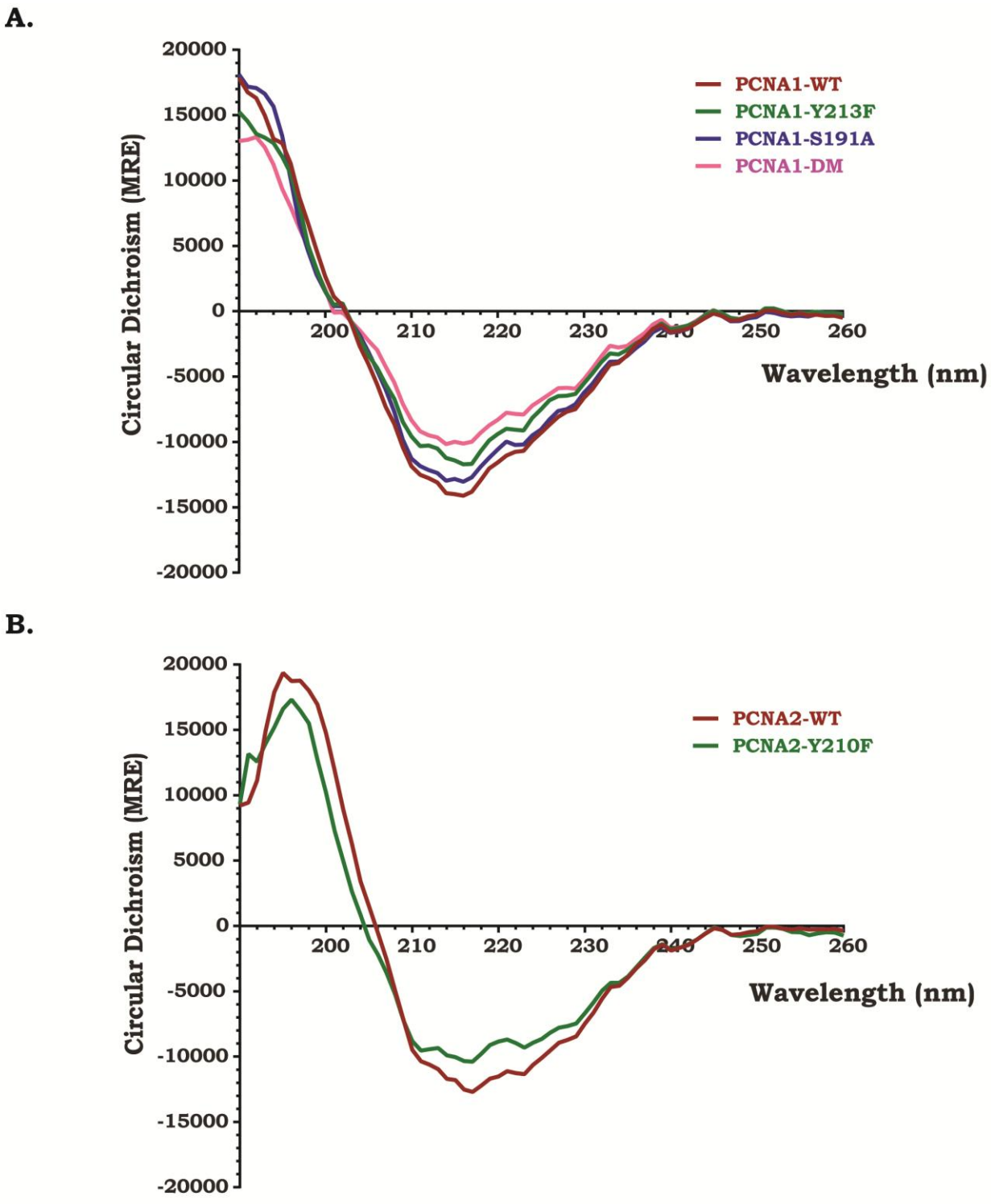



**Figure 3.4. Far UV CD spectroscopy to show the structural integrity of various PfPCNA mutants.** Far-UV circular dichroism spectra of **(A)** PCNA1 wild-type, Y213F-PCNA1, S191A-PCNA1 and DM-PCNA1 and **(B)** PfPCNA2 wild-type and Y210F-PCNA2 were measured in a 1mm path length quartz cell and data were collected at 1.0 nm wavelength resolution using Chirascan Applied Photophysics U.K. S191-PCNA1

showed similar CD spectra as that of PCNA1-WT; however, Y213F- PCNA1 showed a slight upward shift and a greater upward shift in CD spectra was observed for DM-PCNA1. Similarly, CD spectra for PCNA2 **(B)** showed a slight upward shift in CD spectra upon Y210F mutation.

**Table 3.1** α helix and β sheet content corresponding to the CD spectra for wild type PfPCNA proteins and their mutants are shown –

| K2D2 | PCNA1-WT | PCNA1-S191A | PCNA1-Y213F | PCNA1-DM | PCNA2-WT | PCNA2-Y210F |
|---|---|---|---|---|---|---|
| **% α-Helix** | 67.4 | 67.4 | 62.6 | 62.6 | 48.17 | 55.73 |
| **% β-Sheet** | 2.2 | 2.2 | 3.79 | 3.79 | 14.82 | 5.62 |

Further, the CD spectra were quantitatively analyzed for percent α helix and β sheet content using the K2D2 software and are listed in (Table 3.1). We did not observe any change in secondary structure content upon S191A mutation in PfPCNA1. However, there occurred a change in β-Sheet content upon Y213F and Y213F-S191A (DM) mutation of PfPCNA1.

In case of Y210F PCNA2 mutation, changes in both the alpha and beta content of the protein were observed (Table 3.1).

These results suggest similar structural integrity and conformation of WT PCNA1 and S191A PCNA1 mutant. Changes in alpha helix and beta sheet content were observed for Y213F PCNA1, DM PCNA1 and Y210F mutant of PCNA2 compared to their wild type counterparts. The gel filtration results of Y210F PCNA2 mutant protein corroborates with CD data.

**Generation of parasite lines expressing GFP tagged point mutants of PfPCNAs**

We created various point mutant forms of PfPCNA1 [Y213F, S191A and a double mutant with both Y213F and S191A (DM)] and PfPCNA2 (Y210F) by cloning coding regions of corresponding mutant forms of PfPCNA1 & 2 in pARL vector to express them as GFP fusion proteins. 3D7 strain of *P. falciparum* parasites were transfected using

these constructs (including the wild type PCNA1 & 2) as described in the materials and method section. Generation of transfected parasites was confirmed by direct visualization under microscope for GFP signals as well as by western blot analysis using antibodies specific to the PfPCNAs. For this purpose, parasite lysates prepared from unsynchronized parasites from the transfected lines were subjected to immunoblot analysis using antibodies against PfPCNA1 and 2 (Figure 3.5).

Antibodies against PfPCNA1 as well as PfPCNA2 could identify the respective endogenous PCNA protein at the right size (~35 kDa) as well as an additional band at ~55 KDa representing the GFP (~25 kDa) fused PfPCNAs.

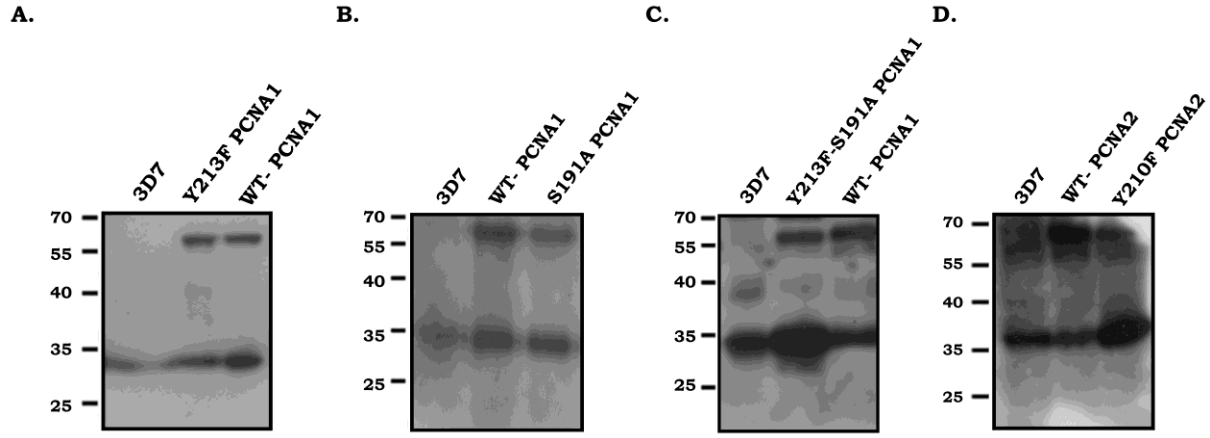



**Figure 3.5. Expression analysis of wild type and point mutants of PfPCNA as GFP-fusion proteins following transfection of the parasites.** Parasite lysates obtained from untransfected 3D7 *P. falciparum* parasites and parasites expressing Y213F PCNA1 **(A)**, S191A PCNA1 **(B)**, Y213F-S191A PCNA1 (DM) **(C)** and Y210F PCNA2 **(D)** along with their wild type forms as positive control were resolved in a SDS-PAGE followed by western blotting using antibodies against PfPCNA1 or PfPCNA2. Results indicate that respective GFP fusion proteins are expressed in all the transfectants but not in untransfected 3D7 control.

**Role of putative phospho-sites on protein stability of PfPCNAs upon DNA damage**

We were curious to know the fate of PfPCNAs containing tyrosine and serine mutant forms in response to DNA damage. Therefore, we performed similar DNA damage experiments by exposing ~36 hrs synchronized parasites expressing mutant PfPCNAs with 10mM HU for 6 hours. We further checked the status of these proteins under DNA damaging conditions by performing western blot analysis using HU untreated and treated parasite lysates. Y213F PCNA1-GFP, S191A PCNA1-GFP and DM PCNA1

proteins showed upregulation in the HU treated parasite lysate as detected by antibodies against PfPCNA1 while no such change was observed in the protein levels of Y210F PCNA2 GFP (Figure. 3.6). However, PfActin remained constant under similar experimental condition. These results suggest that Y213 and S191 may not be involved in stabilization of PfPCNA1 following DNA damage. Since, Y213 and S191 of PfPCNA1 have been shown to be phosphorylated earlier (Treeck, Sanders et al. , Wang, Nakajima et al. 2006), the similar upregulation of the mutant forms like wild type PfPCNAs suggest that phosphorylation of these residues may not have any direct effect on protein stability following DNA damage. In case of Y210F mutation of PfPCNA2, the mutant protein may not trimerize successfully under *in vivo* conditions. This could be the reason for not showing any upregulation similar to wild type PfPCNA2 as shown in previous chapter of this study.

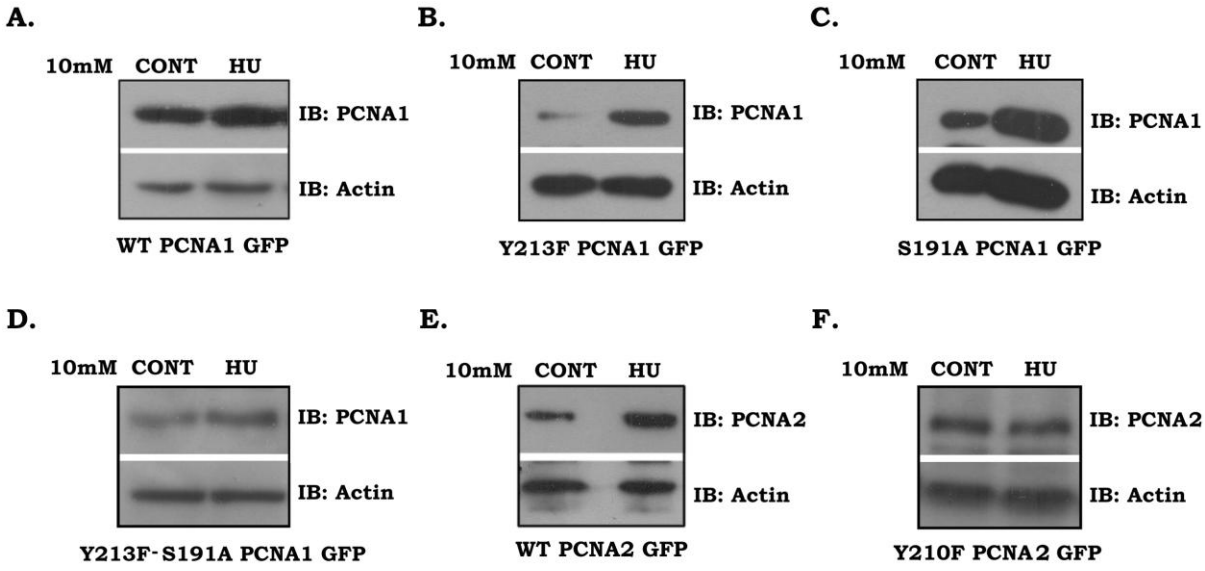



**Figure 3.6. The status of wild type and different mutant forms of PfPCNA1 and PfPCNA2 following DNA damage.** Synchronized trophozoite stage parasites expressing GFP fusion of wild type and various PfPCNA mutant proteins were exposed to 10mM HU for 6 hours and effect on PfPCNAs was checked using antibodies against PfPCNA1 and PfPCNA2 by WB analysis. WT PCNA1 GFP **(A)**, Y213F PCNA1 GFP **(B)**, S191A PCNA1 GFP **(C)**, DM PCNA1 **(D)** and WT PCNA2 GFP **(E)** showed upregulation at protein level in HU treated lane while and Y210F PCNA2 GFP **(F)** did not show any change upon HU treatment. PfActin remained unchanged in each case (loading control).

**Subcellular localization of PfPCNA1 and different point mutants when over-expressed as GFP-fusion proteins in 3D7 *P. falciparum* parasites**

Next, we were interested to look at the *in vivo* localization of PfPCNA1 when mutated at Y213 and S191 residues. The localization of a protein in its cellular environment is the fundamental behavior of any protein. Therefore, we assessed the effect of point mutations at these residues on the subcellular localization of PfPCNA1 with the aid of parasite lines expressing them as GFP fusion proteins. We have already shown in chapter 1 of this study that PCNA1 forms distinct foci associated with the parasite nucleus. We performed live cell imaging analysis for all the parasite lines including WT-PCNA1 GFP, Y213F-PCNA1 GFP, S191A-PCNA1 GFP and DM-PCNA1 GFP lines (Figure 3.7 A-D).

Interestingly, Y213F-PCNA1 GFP expression showed fewer parasites with PCNA1 foci as the majority of the parasites showed diffused GFP signals (Figure 3.7 B), S191A-PCNA1 GFP expressing parasites (Figure 3.7 C) showed nuclear foci in some cells as well as in some parasites protein showed diffused pattern. However, DM-PCNA1 expression showed diffused GFP signals in most of the parasites (Figure 3.7 D). We performed statistical analysis for the number of parasites bearing PCNA1 foci for all the transfectants as shown in **Table 3.2**. For this purpose, more than 100 cells for each parasite line were analyzed. These results suggested a role of Y213 mutation on nuclear pool of PCNA1 since PCNA1 foci bearing parasites decreased drastically in Y213F PCNA1 expressing parasites. DM-PCNA1 GFP expressing parasites showed most dramatic effect on PCNA1 foci formation. However, S191A mutation of PfPCNA1 does not seem to affect the nuclear localization drastically.

**Table 3.2 Statistical analysis of PCNA1 foci bearing parasites expressing WT PCNA1 and different point mutants as GFP fusion protein.**

| Foci bearing parasites | Rings / early trophozoite | Mid trophozoite | Late trophozoite / schizont |
|---|---|---|---|
| **WT PCNA1** | 17.31 % | 45.45 % | 45.94 % |
| **Y213F PCNA1** | 11.32% | 17.64 % | 33.33 % |
| **S191A PCNA1** | 8 % | 26.66 % | 36.17 % |
| **DM PCNA1** | 0 % | 6.25 % | 16.67% |

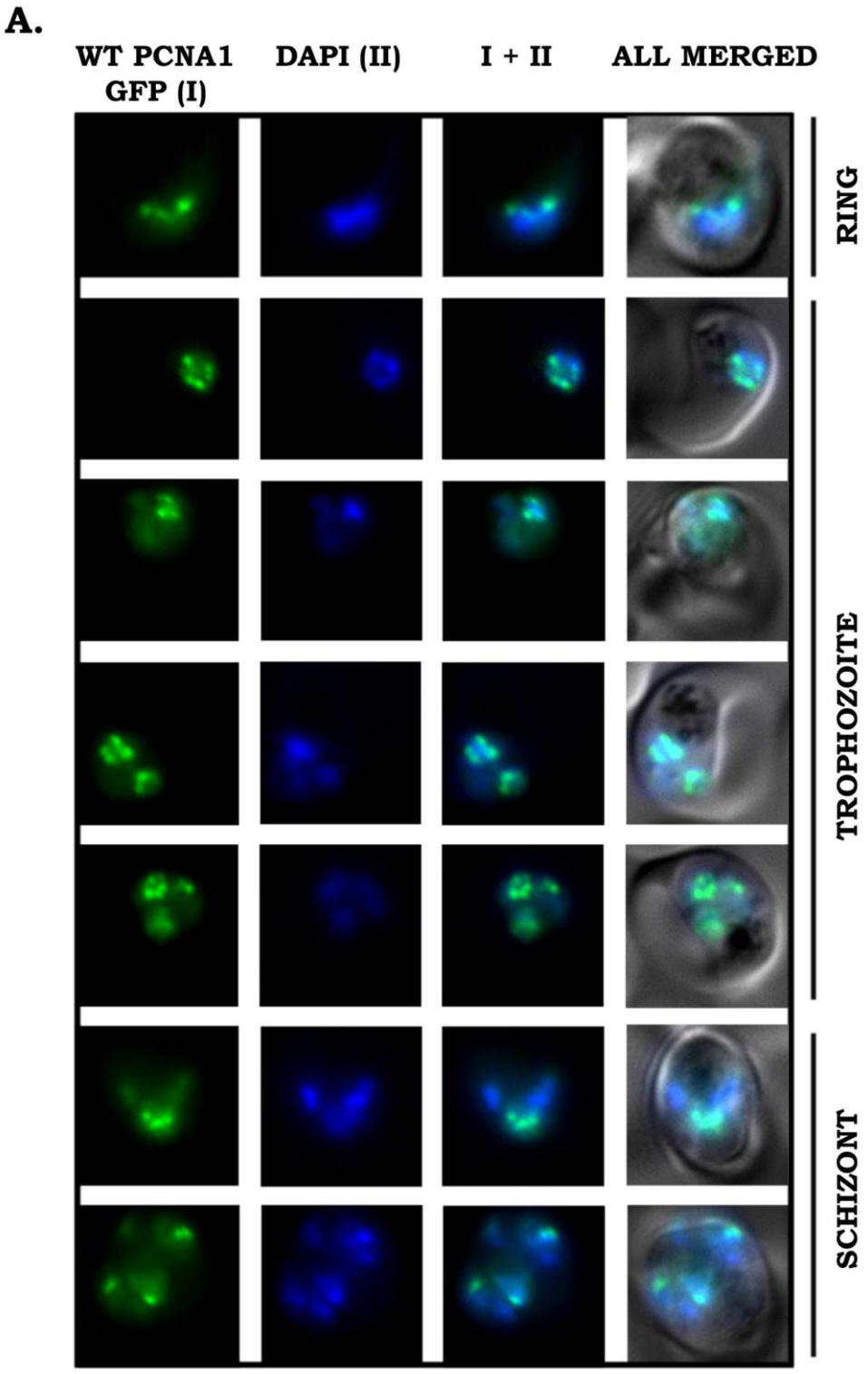



**Figure 3.7. (A) Subcellular localization of WT-PCNA1 GFP by Live cell imaging**. Representative images of PCNA1-WT GFP transgenic parasites with nuclear punctuate foci in various asexual stages of the parasites are shown. At least 100 parasites were counted including Ring, Trophozoite and Schizont stages.

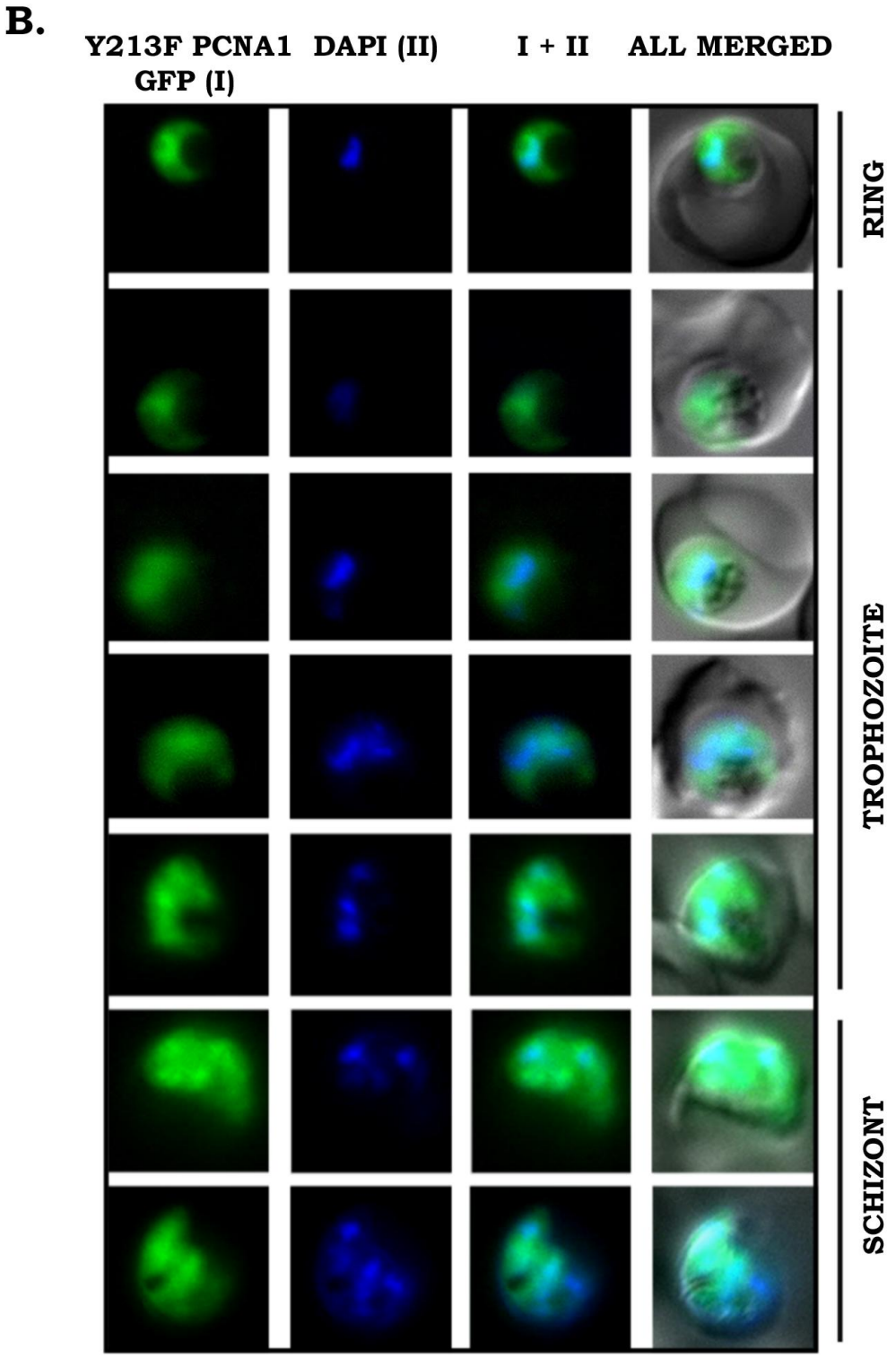



**Figure 3.7. (B) Subcellular localization of Y213F-PCNA1 GFP by Live cell imaging**. Representative images of Y213F-PCNA1 GFP transgenic parasites with diffused nuclear as well as cytoplasmic GFP signals in various asexual stages of the parasites are shown. At least 100 parasites were counted including Ring, Trophozoite and Schizont stages.

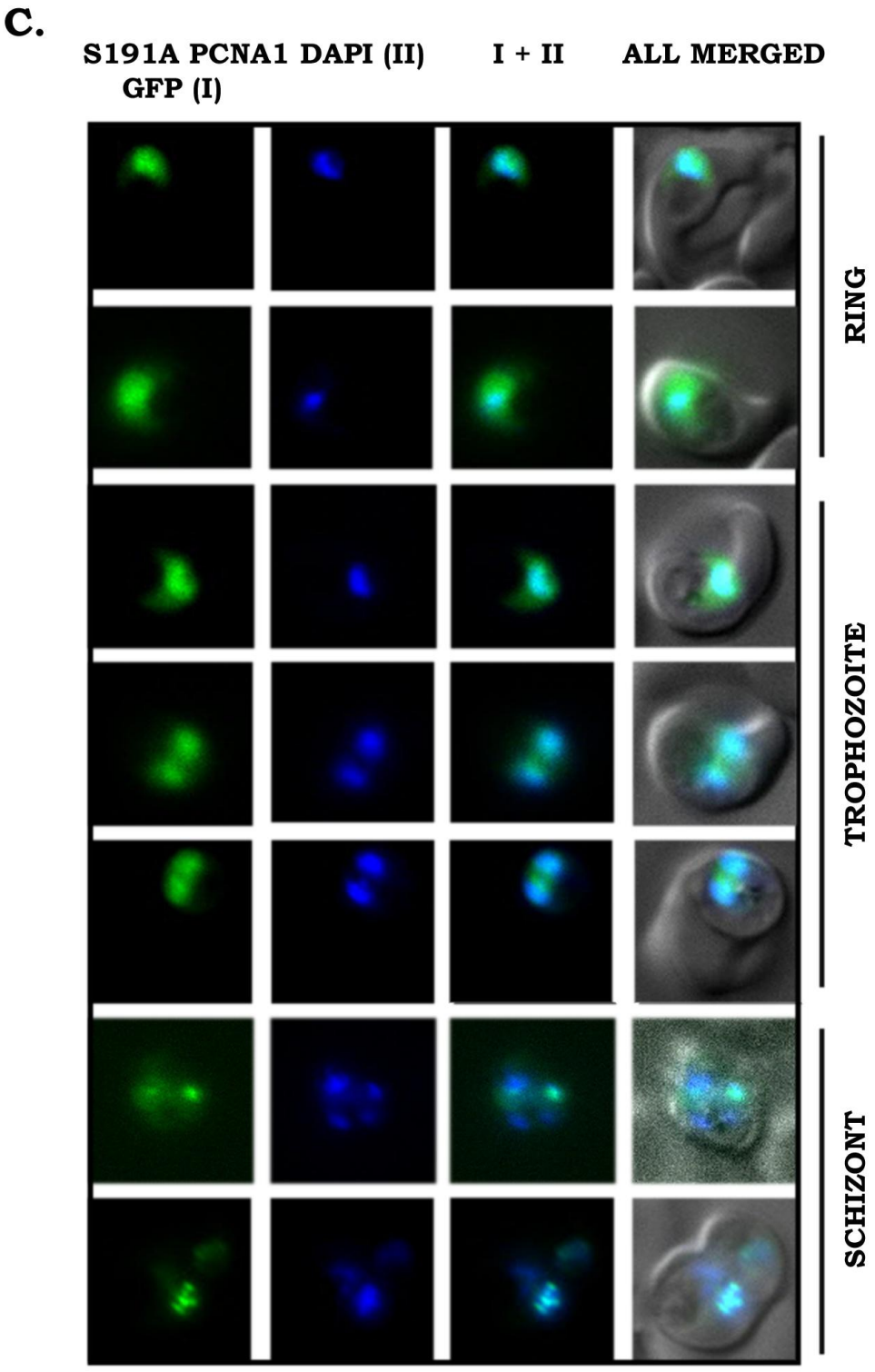



**Figure 3.7. (C) Subcellular localization of S191A-PCNA1 GFP by Live cell imaging**. Representative images of S191A-PCNA1 GFP transgenic parasites with nuclear foci as well as diffused nuclear GFP signals in various asexual stages of the parasites are shown. At least 100 parasites were counted including Ring, Trophozoite and Schizont stages.

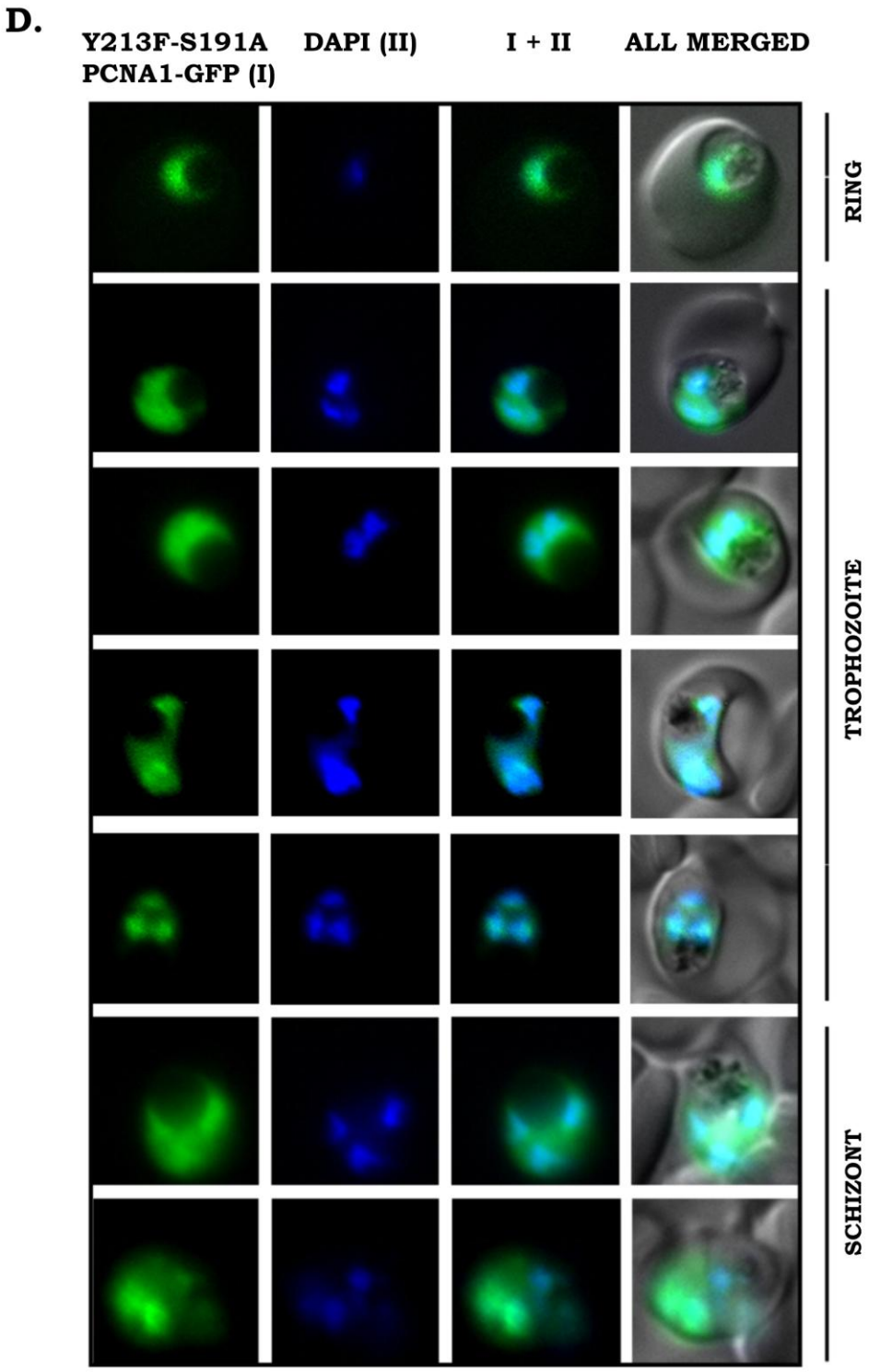



**Figure 3.7. (D) Subcellular localization of Y213F-S191A PCNA1 GFP (DM-PCNA1) by Live cell imaging**. Representative images of DM-PCNA1 GFP transgenic parasites with diffused nuclear and cytoplasmic GFP signals in various asexual stages of the parasites are shown. At least 100 parasites were counted including Ring, Trophozoite and Schizont stages.

**Y213 residue plays a role in chromatin stabilty of PfPCNA1**

We have already mentioned earlier that PCNA is present in cytoplasm as well as in the nucleus. We wanted to confirm the functional relevance of Y213 and S191 residues for PfPCNA1 protein in the parasites over-expressing them as GFP fusion proteins. For this purpose, we performed sub-cellular fractionation experiments and isolated the cytoplasmic (CF), nuclear-soluble (NsF) and nuclear insoluble fractions (NiF) from the transgenic parasites expressing WT-PCNA1 GFP, Y213F-PCNA1 GFP, S191A-PCNA1 GFP and DM-PCNA1 GFP as described in the materials and methods section. The fractions were then electrophoresed and subjected to Western blot analysis using antibodies specific to PfPCNA1.

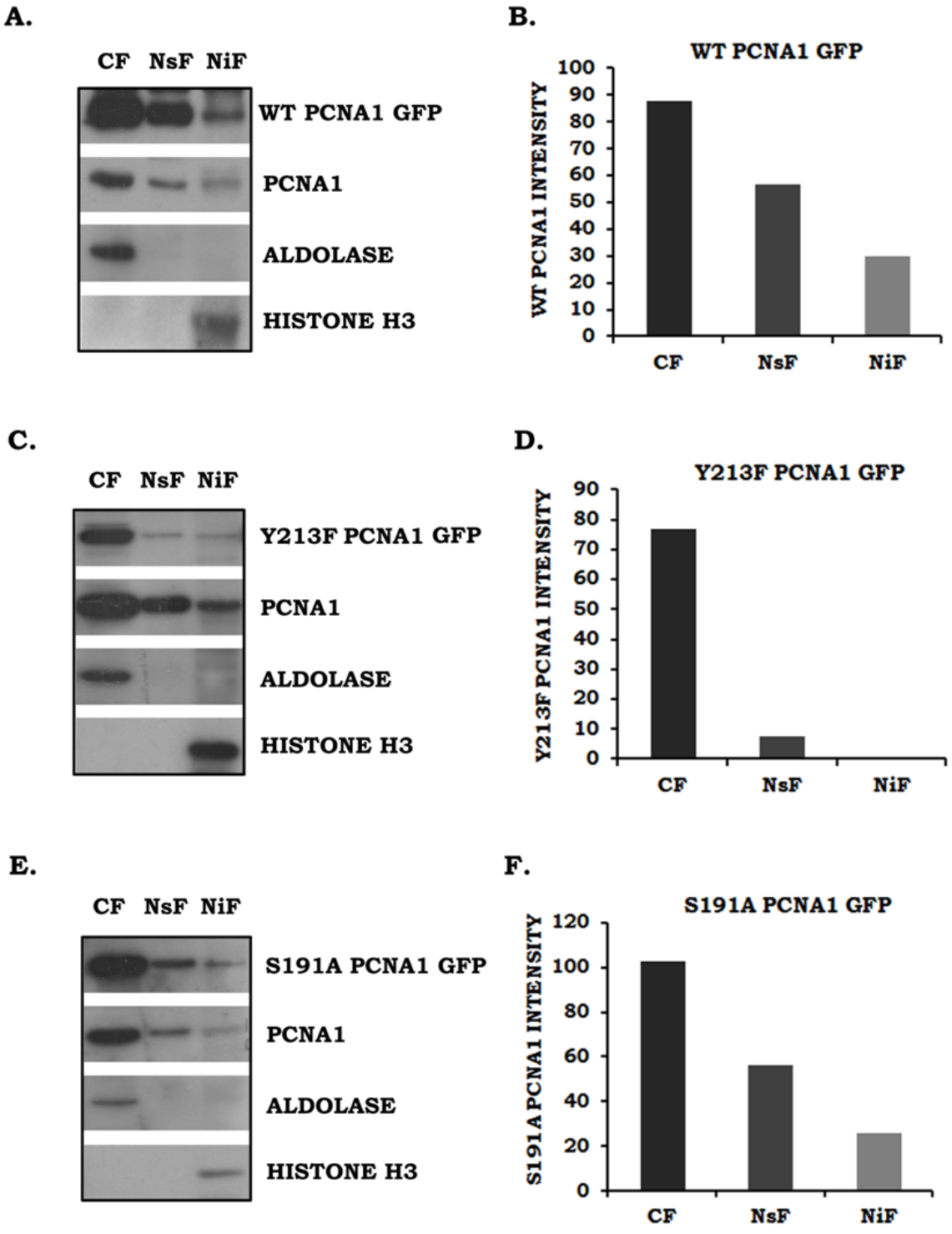
Interestingly, we found that as compared to WT-PCNA1 GFP (Figure 3.8 A-B) the presence of Y213F mutant is reduced drastically in nuclear fraction (combining nuclear soluble and insoluble pools) and the protein was present mostly in cytoplasmic pool as shown in Figure 3.8 C-D. The endogenous PCNA1 in the same parasites was present both in nuclear soluble and nuclear insoluble fractions apart from a cytoplasmic pool suggesting effect on nuclear retention and chromatin stability of PfPCNA1 upon Y213F mutation. This is in corroboration with what is observed in the case of HsPCNA Y211 mutation, where Y211 residue has a role in chromatin stability of PCNA (Wang, Nakajima et al. 2006).

However, this was not the case for S191A-PCNA1 GFP protein since it showed reasonable amount of protein in nuclear (combining nuclear soluble and insoluble pools) similar to that of endogenous PCNA1 (Figure 3.8 E-F) in the same parasites as well as to that of WT-PCNA1 GFP expressing parasites (Figure 3.8 A-B).

DM-PCNA1 GFP expressing parasites showed mostly cytoplasmic pool of the mutated PCNA1 (Figure 3.8 G-H).

In each case, Histone H3 (used as a marker for nuclear insoluble fraction) could be seen only in nuclear insoluble fractions. Similarly, PfAldolase (used as a marker for cytoplasmic fraction) could be seen in the cytoplasmic fraction only; validating fractionation experiment.

These results suggest a role of Y213 in nuclear retention and chromatin stability of PfPCNA1 protein. However, S191 residue may not be involved in chromatin stability and may serve function relevant to the PfPCNA1 protein with respect to its various purposes other than DNA metabolism.



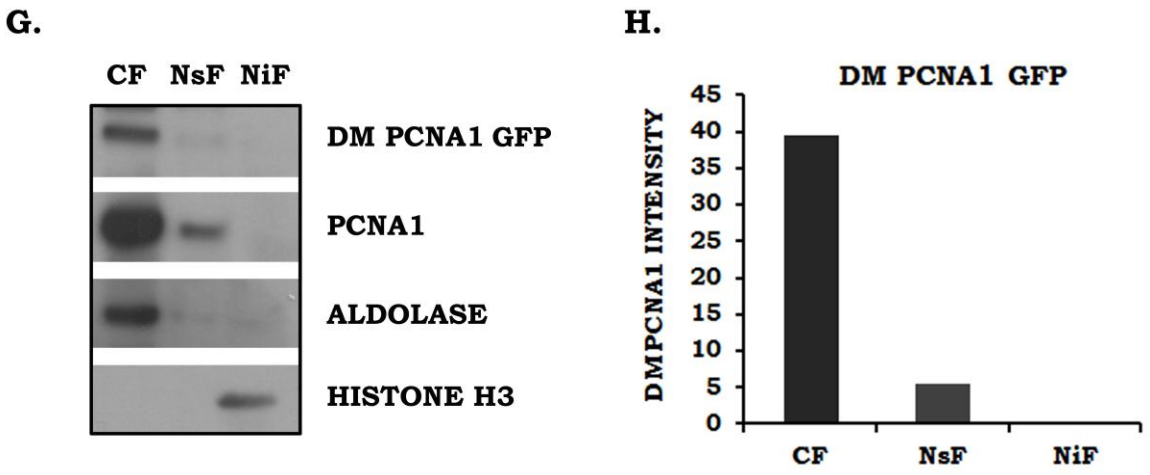



**Figure 3.8. Sub-cellular fractionation of WT-PCNA1 GFP, Y213F-PCNA1 GFP, S191A-PCNA1 GFP and DM-PCNA1 GFP over-expressing parasites.** Cytoplasmic, nuclear soluble (NsF) and nuclear insoluble (NiF) fractions were isolated from parasites over-expressing WT PCNA1 and its various mutant forms as GFP fusion proteins. **(A, C, E, G)** Western blotting with antibodies specific to PfPCNA1 showed the relative presence of endogenous as well as over-expressed GFP fusion proteins in each of the sub-cellular compartments. Histone H3 is used as a marker for chromatin fraction (NiF) and PfAldolase is used as a marker for cytoplasmic fraction (CF). Graphical representation **(B, D, F, H)** for the presence of GFP fusion WT or mutant PCNA1 protein in each sub-cellular fraction by densitometric analysis of the band intensities in corresponding **(A, C, E, G)** western blots using Image J software.

**Loss of PfPCNA1 function occurs upon Y213F mutation**

Previously, we performed parasite survival assay to assess the significance of PfPCNAs over-expression in rescuing the parasites from the DNA damaging effects of HU and MMS.

Here, to investigate the importance of Y213 and S191 residues for PCNA1 function, we performed similar *in vitro* survival experiments. For this purpose, the parasites over-expressing PfPCNA1-WT, Y213F-PCNA1 and S191A-PCNA1 as GFP fusion proteins were treated with the genotoxic agents HU and MMS (at a concentration of 2.5mM HU and 0.002% MMS, respectively) and incubated for 6 hours. The parasites were then washed and allowed to grow in fresh medium for the next cycle. Giemsa staining of the parasites recovered at different time points was performed for the untreated and drug treated parasites as described in the materials and methods section.

We observed differential survival of the parasites in each case. We found that with respect to PCNA1-WT GFP parasite (P = 0.0331, 0.0155 for HU and MMS, respectively) both Y213F-PCNA1 GFP (P = 0.0331, 0.0155 for HU and MMS, respecti-

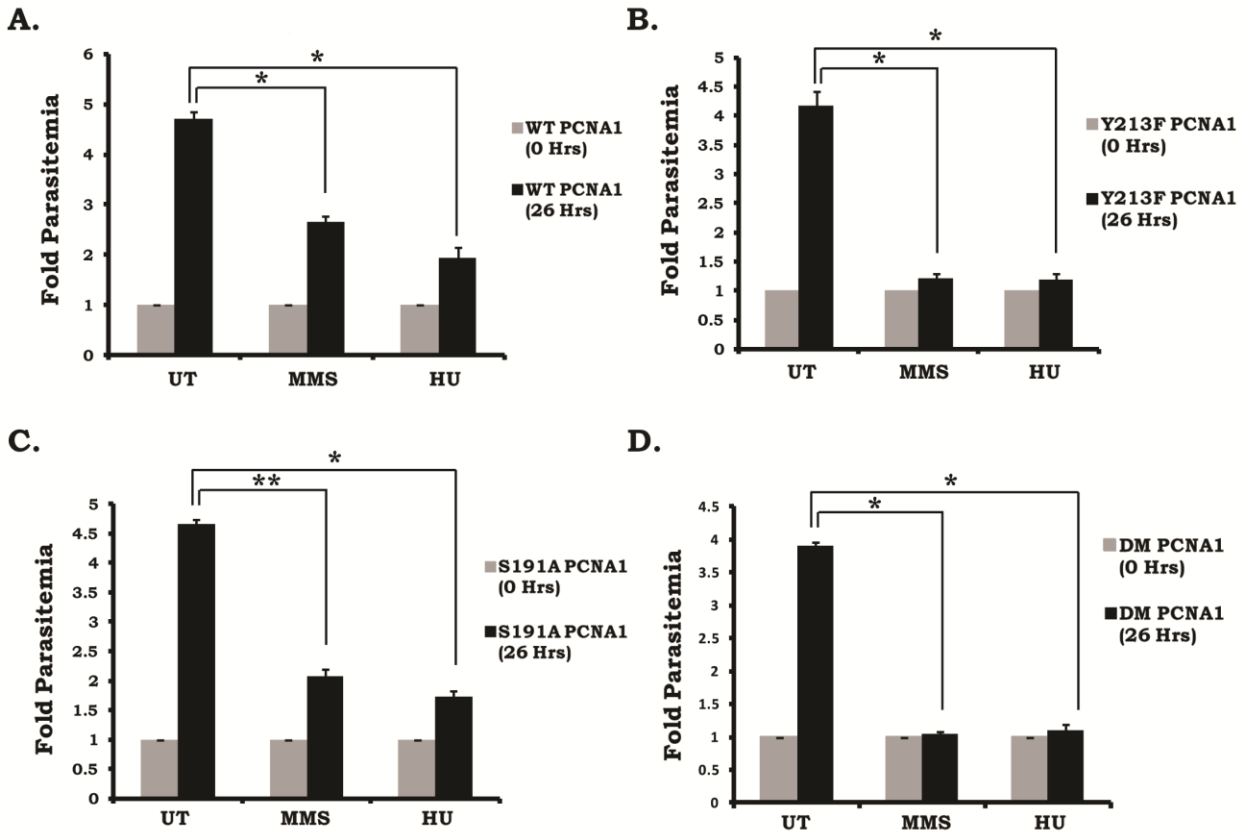



**Figure 3.9. *In vitro* parasite survival assays in presence of DNA damaging conditions upon over-expression of mutant forms of PfPCNA1.** Synchronized replicating stage *P. falciparum* parasites over-expressing WT PCNA1 **(A)**, Y213F PCNA1 **(B)**, S191A PCNA1 **(C)** and Y213F-S191A PCNA1 **(D)** as GFP fusion proteins were maintained at 1% parasitemia and treated with 0.002% MMS or 2.5mM HU for 6 hours. At the end of the treatment, parasites were washed once with complete RPMI media and resuspended in fresh complete RPMI media and followed for different time intervals. At the end of each time point, Giemsa stained slides were prepared and parasitemia was counted. At least 700-800 cells were counted for each treatment and each experiment was done in triplicates. Graphs were plotted for fold increase in the parasitemia for each under control vs. HU/MMS treated condition. Error bars represent SEM of three independent experiments. * represents statistical significance for HU and MMS treated PCNA1-WT GFP parasite (P = 0.0331, 0.0155 for HU and MMS, respectively), Y213F-PCNA1 GFP (P = 0.0331, 0.0155 for HU and MMS, respectively), S191A-PCNA1 (P = 0.0162, 0.0065 for HU and MMS, respectively) and DM-PCNA1 GFP (P = 0.0262, 0.0274 for HU and MMS, respectively) compared with respective untreated counterparts. (*P < 0.05 and **P < 0.01) (Student's t test).

-vely) and DM-PCNA1 GFP (P = 0.0262, 0.0274 for HU and MMS, respectively) parasite lines could not recover the parasites from damaging effects of the drugs (Figure 3.9 A, B, D) and showed a response similar to untransfected 3D7 parasites (Figure 2.6-8, Chapter 2) suggesting for a loss of functional activity of PCNA1 due to Y213F mutation. While S191A-PCNA1 over-expression resulted in moderate overcome of the damaging effects of HU and MMS (P = 0.0162, 0.0065 for HU and MMS, respectively) (Figure 3.9 C). These results suggest that S191 mutation does not affect PCNA1 function as compared to Y213 mutation.

**Identification of the kinase responsible for the phosphorylation of PfPCNAs**

*In vitro* kinase assays using parasite extracts as a source of kinases have already demonstrated phosphorylation of PfPCNAs (Mitra P, Dhar laboratory, unpublished data). To establish *in vivo* phosphorylation of PfPCNAs, we tried immunoblotting PfPCNA1 or PfPCNA2 immunoprecipitates using phophospecific antibodies. However, phosphotyrosine and phosphoserine specific antibodies procured commercially did not give promising results inspite of successful immunoprecipitation of the respective proteins. There may be a small pool of phosphorylated PfPCNAs which remained undetectable by the phosphospecific antibodies.

We were interested in identifying the specific kinase that may be responsible for phosphorylation of PfPCNAs. In search of a kinase for PfPCNAs, we performed immunoprecipitation using antibodies specific to GFP or PfPCNA2 to pull down immune complexes of PCNA1-GFP (form total extracts using parasites over-expressing PCNA1-GFP) or PfPCNA2 (form total extracts using 3D7 parasites), respectively followed by LC-MS/MS for identification of the interacting partners of PfPCNAs as discussed in Chapter 2. We failed to obtain any kinase in immunoprecipitation reactions of PfPCNAs (data not shown). This may be due to the presence of a huge cytoplasmic pool of PfPCNAs where the protein may be present in a non-phosphorylated state. Also, the kinase-substrate interactions are often very transient making it difficult to isolate such complexes.

A study involving PCNA protein turnover showed that HsERK8 (extracellular signal-regulated kinase 8) is required for PCNA protein stability. Chromatin-bound HsERK8 interacts via its PIP motif with PCNA (chromatin form). Silencing HsERK8 resulted in an increased recruitment of HDM2 which in turn decreased PCNA levels and increased DNA damage (Groehler and Lannigan 2010). A *Plasmodium* homolog of ERK8 was found in *P. falciparum* (PF3D7_1431500 MAP1).

The CDK (cyclin-dependent kinase) family of protein kinases are also involved in the regulation of the cell cycle in eukaryotes. PfPK5 (*Plasmodium falciparum* protein kinase 5) with 60% identity with human cdc2, is one of the Pf CDKs to display promiscuity in its cyclin requirement, i.e., it is active (upon autophosphorylation) in the presence of Pfcyclin1 and 3 as well as in the presence of RINGO, a *Xenopus* protein unrelated to cyclins. RINGO activates a subset of mammalian CDKs (Graeser, Wernli et al. 1996, Merckx, Le Roch et al. 2003). *Plasmodium falciparum* has 4 cyclin partners for PfPK5, out of which Pf cyclin1 and 3 have been demonstrated for PfPK5 activation and interestingly cyc 1 and cyc 2 carries the PCNA interacting peptide (PIP) motif as well. Moreover, the expression levels of PfPK5 and cyclin 1 peak at the trophozoite stage that coincides with DNA replication suggesting their involvement during DNA replication.

It is possible that PfERK8 and/or PfPK5 are the putative kinases for PfPCNAs. In order to test this, we performed preliminary experiments like *in vitro* kinase assay using recombinant purified kinases including PfERK8 and PfPK5 and wild type PfPCNAs as substrates.

**Cloning, Expression and Purification of PfERK8 and purification of PfPK5 and RINGO**

In PlasmoDB.org, an ORF (PF3D7_1431500 MAP1) was identified that could be a possible homolog of ERK8. It is 2745 bp long and encodes for 914 amino acids residues corresponding to 107.279 kDa protein.

Kinase domain of PfERK8 (366 amino acid, 42.37 KDa protein) was amplified by Polymerase Chain Reaction (PCR) using specific primer pair (Table M3 in materials and methods section) and *P. falciparum* genomic DNA as template (Figure 3.10 A) and cloned subsequently into *BamHI- XhoI* sites of pET-28a vector. Transformation was car-

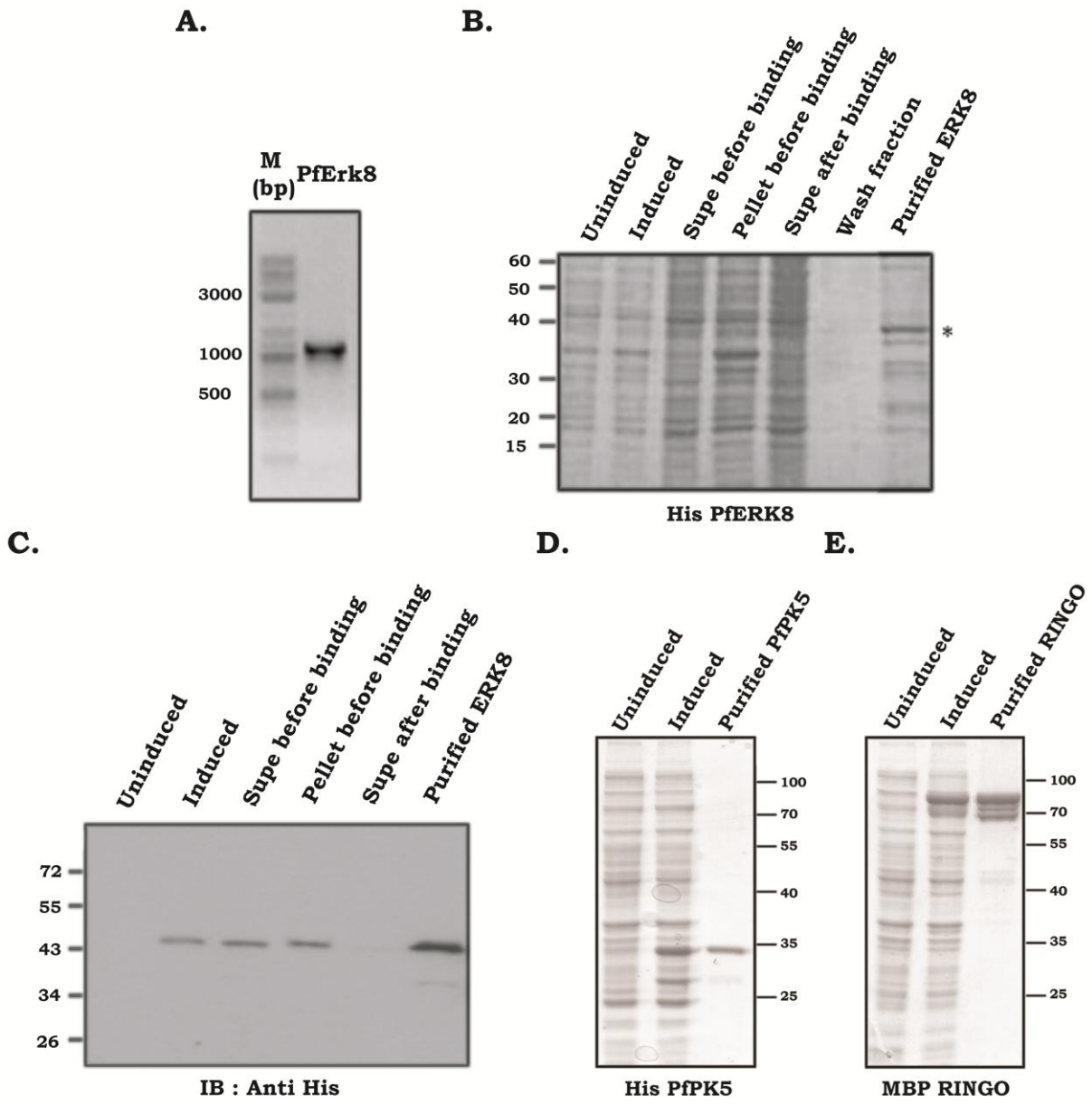



**Figure 3.10. PCR amplification (*Pferk8*), expression profile and protein purification profile of PfERK8, PfPK5 and RINGO. (A)** PCR amplification of *Pferk8* (kinase domain) using specific primers and *P. falciparum* genomic DNA template. **(B)** Coomassie stained gel shows purification profile of His-PfERK8 using Ni-NTA affinity chromatography **(C)** Western blot analysis using antibodies against $His_6$ confirmed expression and purification of $His_6$ PfERK8 protein. Bacterial lysates prepared from IPTG induced *E.coli* transformed with respective PfERK8 constructs showed specific signals, while the uninduced lane showed no such signal. M-marker (kDa) depicts protein markers while M (Kb) depicts DNA markers. Asterisk (*) shows the purified protein. **(D, E)** Coomassie stained gel showing purification of $His_6$-PfPK5 and MBP-RINGO proteins.

-ried out with the recombinant constructs using *E.coli* BL21 codon plus cells for protein expression and purification (Figure 3.10 B). The expression and purification of $His_6$ tagged PfERK8 protein was further confirmed using anti-$His_6$ antibodies (Figure 3.10 C).

Cloning of PfPK5 and RINGO has already been reported earlier (Merckx, Le Roch et al. 2003). We purified the full length $His_6$ PfPK5 from *E.coli* BL21 codon plus cells by Ni-NTA affinity purification. RINGO was cloned as MBP fusion protein and purified using amylose resin as described in material and method section (Figure 3.10 D, E). Purified proteins were further dialyzed and used for *in vitro* kinase assays.

***In vitro* Kinase assays using PfERK8 and PfPK5 as kinase and PfPCNAs as substrate**

In order to check whether PfERK8 and PfPK5 serve as the kinase for PfPCNAs phosphorylation, we performed *in vitro* kinase assays using recombinant purified proteins (both kinase and substrate) as described in material and method section. In the same assay MBP (Myelin basic proetein) or Histone H1 were used as the positive control for PfERK8 or PfPK5 based kinase assays, respectively. The results indicated that MBP or Histone H1 was phosphorylated in the presence of PfERK8 or PfPK5-RINGO, respectively suggesting the purified proteins was active as kinases. However, PfPCNA1 & 2 could not be phosphorylated under the same conditions suggesting inability of both PfERK8 and PfPK5 to phosphorylate PfPCNAs. It is possible that some other kinases are required for the phosphorylation of PfPCNAs.

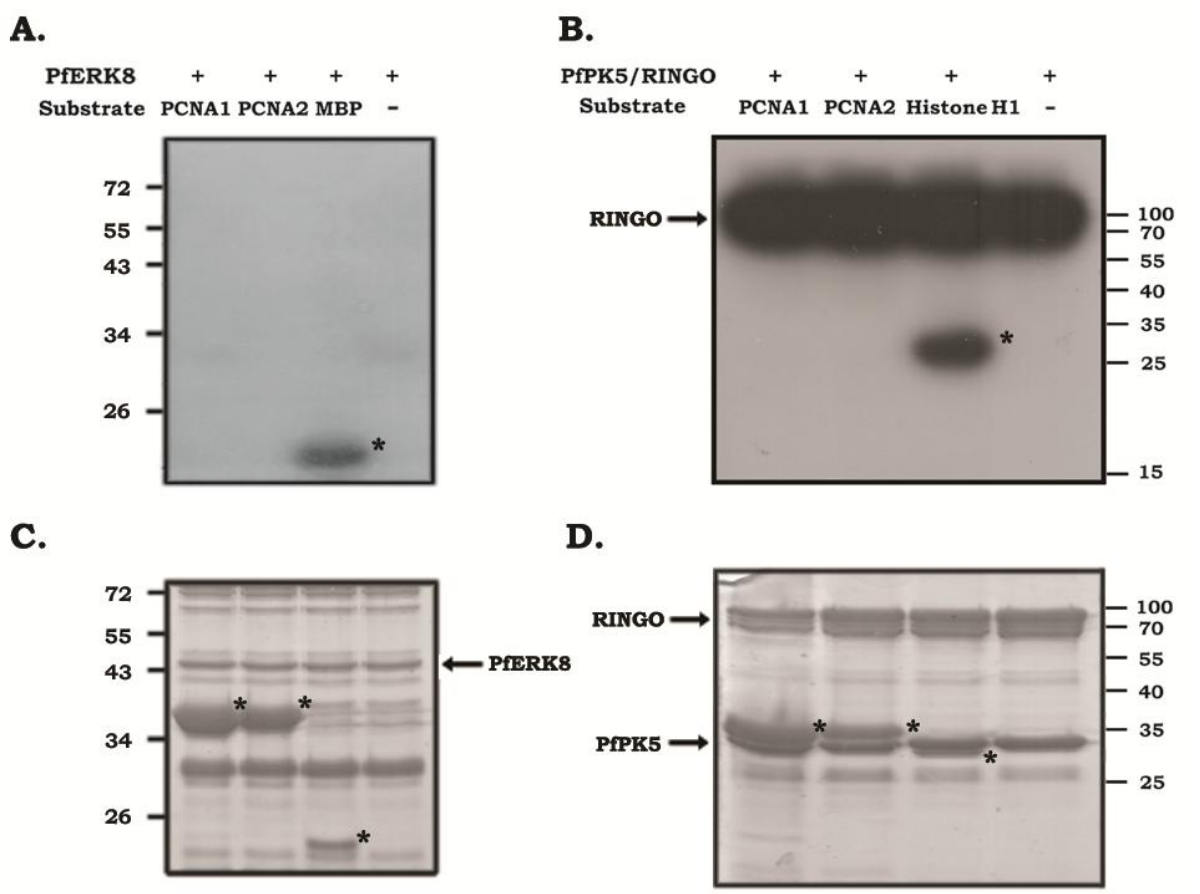



**Figure 3.14.** ***In-vitro*** **phosphorylation of PfPCNA1 and 2 proteins. (A, B)** Purified His-PfERK8 and His-PfPK5 proteins were able to phosphorylate MBP and Histone H1, respectively (positive control), while PfPCNA1 and PfPCNA2 were not phosphorylated under the same experimental conditions, * shows the phosphorylated substrate as indicated in each lane. **(C, D)** Coomassie stained gels are shown as loading control for A and B, respectively. * shows the presence of substrate as indicated in each lane, while → indicates the recombinant kinase used in each assay.

## DISCUSSION

Post translational modifications are essential for proper cellular functions as they play an important role by regulating protein functions. PCNA has been shown to undergo various post-translational modifications (PTMs) including ubiquitination, sumoylation, acetylation and phosphorylation for its regulation in mammalian and yeast systems (Moldovan, Pfander et al. 2007).

In this study, we have made an attempt to understand the role of one such PTM in relation to the role of PfPCNAs in DNA replication and DNA damage response. Wang

*et al.* have shown that Y211 residue of mammalian PCNA plays significant role in chromatin stability of PCNA upon phosphorylation. This tyrosine residue is conserved in both PfPCNA1 and PfPCNA2. Another phosphoproteome study of schizont stage of *P. falciparum* by Treeck *et al*. has reported S191 residue in PfPCNA1 to undergo phosphorylation. Based on the available literature, we considered the importance of these residues for phosphorylation mediated regulation of PCNA stability onto chromatin following DNA damage. We generated point mutant forms of the Y213 and S191 residues in PfPCNA1 and Y210 residue in PfPCNA2.

We initiated our study by assessing the effect of these mutations at the level of protein structure and integrity. Trimerisation is an intrinsic behavior of a processivity factor as it allows it to form a ring like structure to slide freely onto replicating strand of DNA while interacting with the DNA polymerases at the same time.

Gel filtration chromatography results have shown that neither Y213F nor S191A mutation alone or in combination led to a change in trimerisation property of PfPCNA1. Y210F-PCNA2 protein showed trimerisation similar to wild type PCNA2, but there was a fraction of the protein that showed low molecular weight oligomeric forms of the mutant protein probably of a dimer or unstable trimer (~58 KDa, peak corresponding to ~12 ml) resulting upon Y210F mutation of PCNA2.

Far UV CD analysis of all the mutant PfPCNA proteins gave us information about their molecular conformation and secondary structure content. There was no considerable change in CD spectra as well as alpha and beta helical content upon S191A mutation. However, there is a moderate increase in beta sheet content and decrease in α helix content upon Y213F mutation. However, such a change in molecular conformation of the protein did not affect the intrinsic trimerisation behavior of the protein as concluded from gel filtration results. Double mutant at Y213 and S191 of PfPCNA1 retained its trimerisation property but showed a similar change in beta sheet content as observed upon Y213 mutation alone.

Y210F mutation of PfPCNA2 resulted in a significant change both in alpha and beta helix content, which may be the cause of loss of trimerisation to some extent leading to the formation of other oligomeric forms by the mutant protein.

The gel filtration and CD data analysis suggest that different PfPCNA1 mutations may not affect the overall conformation of the protein significantly and in turn the function of PCNA1.

We speculated that phosphorylation would be the probable PTM of PfPCNAs for its modulation in response to DNA damage. We successfully generated various transgenic parasite lines over-expressing these point mutant forms of PfPCNAs as GFP tagged proteins. However, the mutant forms of PfPCNA1 showed similar upregulation following DNA damage like wild type proteins suggesting that these mutations might not have critical role in stability of PfPCNA1 post DNA damage.

The mutant from of PCNA2 did not show upregulation following DNA damage. However, as shown by the gel filtration and CD results, the mutant form may not be structurally stable as the wild type protein that may account for the similar protein level before and after DNA damage.

Previous studies from our laboratory has demonstrated PfPCNAs to undergo phosphorylation by parasite kinases and diminished phosphorylation of recombinant PfPCNA proteins with Y213F/Y210F and S191A mutation.

Further, it became imperative for us to study the *in vivo* effect of the over-expression of the mutant forms with respect to the wild type protein using the transgenic parasite lines. We did not observe any change in the proliferation profile of parasites expressing mutant forms of PfPCNA1.

Live cell imaging studies using parasites expressing GFP-tagged wild type and mutant PfPCNA proteins showed significant effect of Y213F mutation on localization of the protein. Y213F-PCNA1 protein lost its ability significantly to form distinct foci, while S191A mutation did not show much change in PCNA1 foci distribution in the parasites. Also, DM-PCNA1 showed mostly diffused localization pattern. We complemented our study by performing sub-cellular fractionation experiments and found Y213F-PCNA1 and DM-PCNA1 to shuttle their nuclear pools to the cytoplasmic compartments of the parasites. However, S191A mutation of PfPCNA1 did not lead to a significant change in its nuclear pool compared to the wild type protein.

Inspite of retaining the trimerisation property, we observed that Y213F mutation of PfPCNA1 led to the destabilization of PfPCNA1 from the chromatin and lacked

formation of characteristic PCNA1 foci associated with the parasite nucleus. Loss of a phospho-site in the protein may be the cause for such a behavior. However, at present we could not provide direct evidence of *in vivo* phosphorylation of PfPCNA1 at Y213 residue inspite of several attempts of immunoprecipitation experiments followed by immunoblotting with phospho-specific antibodies or vice versa. However, our results were in complete corroboration with the finding in mammalian cells which formed the basis of this objective of our study. It has been shown that phosphorylation of Y211 residue of HsPCNA leads to its chromatin stability (Wang, Nakajima et al. 2006). Probably, due to lack of a phosphosite at Y213 residue and subsequent loss of phosphorylation of PfPCNA1 resulted in destabilization of chromatin bound Y213F PCNA1 GFP. These results highlight the evolutionary conservation of PCNA function in eukaryotes.

Rescue experiments using transgenic parasite lines over-expressing phospho-mutants of PfPCNA1 following DNA damage also showed the inability of Y213F-PCNA1 and DM-PCNA1 to overcome the effect of DNA damage.

Altogether, these results suggest the importance of Y213 residue in PfPCNA1 function by means of chromatin stability upon phosphorylation.

Phosphorylation at S191 residue of PfPCNA1 has been shown in schizont stage parasites. Previous *in vitro* kinase assays showed diminished phosphorylation using S191A-PCNA1 as substrate compared to WT PCNA1 (Mitra P, Dhar laboratory, unpublished data). Moreover, genetic complementation of yeast deletion mutant of *pcna* with S191A-PfPCNA1 mutant form resulted in slow growing yeast cells. It is clear from the previous data and evidences from present study that loss of phosphorylation of S191 residue of PfPCNA1 did not result in a loss of chromatin stability and not much phenotypic alteration in terms of localization were observed by live cell imaging or sub-cellular fractionation results. Over-expression of S191A PCNA1 could rescue the parasites to some extent following DNA damage as it was in case of WT PCNA1. Therefore, S191 mutant of PCNA1 may not lose its function *in vivo*. However, at this stage the relevance of this residue with respect to phosphorylation cannot be explained.

Apart from serving as a processivity factor for DNA polymerases, PCNA plays a diverse range of functions within a cell. Recently Wu *et al.* have shown that there is a novel

sensing system (an equivalent of the two-component regulatory system of bacterial signal transduction) and some potential regulation mechanisms by which parasites respond to external stimuli including inflammatory cytokines, such as TNF. It has been suggested that HSP90 could be a component of a membrane-bound histidine kinase that receives the input stimuli from some uncharacterized external receptors, and reversible phosphorylated PfPCNA1 could be a response regulator resulting in change in cellular physiology. It has been shown that PfPCNA1 undergoes triple phosphorylation at S87, S91 and S92 residues by mass spectrometry identifications from phospho-peptide enrichment. The study also suggested that this sensing system might also be shared with cell cycle checkpoints. The stimulus which triggers PCNA1 dephosphorylation/degradation may have an implication in DNA replication stress and damage (Wu. *et al,* in 2016). Another study has shown that *in vivo,* differential phosphorylation of proteins occurs between schizonts and merozoites (Lasonder, Green et al. 2015) and PfPCNA2 to carry a phosphosite at S203 residue. We could not verify the phosphorylation status of these residues or their effect following mutation in our studies. In summary, we have analyzed the importance of the conserved tyrosine residues (Y213 in PCNA1 and Y210 in PCNA2) and S191 residue in PfPCNA1 for *in vivo* function of PfPCNAs. We find that the mutation of tyrosine residue in PfPCNAs compromises their function but serine mutation may have very limited effect. S191 phosphorylation has been reported in phosphoproteome data but there is lack of direct experimental evidence for tyrosine phosphorylation. However, conserved nature of this tyrosine residue in other species including humans where it has been reported to be phosphorylated suggests its conserved function in *P. falciparum.* The mutation of tyrosine residue does not affect its trimerisation property and overall conformation drastically. Therefore, the inability of tyrosine mutant to perform its *in vivo* function may be contributed to the lack of its phosphorylation, although it needs to be verified experimentally. It is possible that tyrosine phosphorylation is indeed required for PfPCNA nuclear retention and *in vivo* function. Alternatively, tyrosine mutant proteins may not interact properly with their interacting partners leading to the loss of *in vivo* function.

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# SUMMARY

# Summary

*Plasmodium falciparum* causes one of the most dreadful diseases to mankind known as malaria. Typical signs of malaria include fever, fatigue, anaemia etc. eventually leading to death. 198 million cases of malaria were estimated worldwide in 2013 and 5, 84, 000 deaths occurs mostly in poor, tropical and subtropical areas of the world. Further, 3.4 billion people across 106 countries and territories were estimated at a risk of malaria, which is nearly half of the world's population. The genome of *P. falciparum* has been published in 2002 (Gardner, Hall et al. 2002) which paved the way for better understanding of parasite biology and pathology. The genome sequence of *P. falciparum* reveals some striking features e.g. high AT content (overall 80.6% AT); several single rather than long tandemly repeated arrays of ribosomal RNA (rRNA) genes, (18S-5.8S-28S); extensive redundancy in tRNA genes; considerable variation in the chromosomes length (especially subtelomeric region); complex subtelomeric repeat structure. The *P. falciparum* genome contains at least some components of DNA replication, repair and recombination processes similar to other eukaryotes.

The life cycle of *P. falciparum* is complex. It is completed in two different hosts, human and mosquito and involves invasive, trophic and replicative forms. A pathogen's success relies on its ability to multiply and propagate in its host, which in turn, depends on an efficient DNA replication process and successful immune evasion. Exploring different aspects of *P. falciparum* biology to understand various underlying mechanisms of the disease pathogenesis has become an urgent need. Studying DNA replication offers scope and hope to combat such a deadly disease. We have focused on replication proteins of *P. falciparum,* and particular interest of my study is to investigate the role of processivity factors in DNA replication and DNA damage response, namely proliferating cell nuclear antigens (PCNA). Interestingly, unlike most other eukaryotes, *P. falciparum* possesses two homologues of PCNA, i.e. PCNA1 and PCNA2.

Role of PCNA has been well documented in DNA replication and it has been widely accepted as a marker of replication fork progression (Leonhardt, Rahn et al. 2000). Previous studies including genetic complementation of *ScPOL30* deletion mutant and formation of distinct replication foci by PfPCNA1 have substantiated its active

participation in DNA replication and to play an important role of primary processivity factor in *P. falciparum* (Mitra, Banu et al. 2015). These observations are also consistent with those found in apicomplexan *T. gondii* where TgPCNA1 appears to be the major replisomal PCNA (Guerini, Behnke et al. 2005).

As mentioned above, presence of two PCNAs in *P. falciparum* led us to explore the possibility of formation of heterotrimers. Co-immunoprecipitation and co-immunofluorescence data along with previous pull down assays suggest that PCNA1 and PCNA2 primarily form homotrimers. However, a weak or transient interaction between the two cannot be ruled out, suggesting formation of PCNA heterotrimer formation in the parasite nucleus. A recent study on mass spectrometric analysis of PCNA1 immunoprecipitated samples has shown the presence of PCNA2, also support PCNA heterotrimers formation in *P. falciparum* (Wu, Cruz et al. 2016). PfPCNA2 co-immunoprecipitation upon MS-analysis of the PCNA1 immune complexes in a recent study by Wu, *et al.* further supports our findings of PCNA heterotrimers formation in *P. falciparum.* The lack of evidence for a strong heterotypic interaction likely explains the independent sub-cellular localization patterns of each of the PfPCNAs and stage dependent interaction can also be one possibility.

Both the PfPCNAs show their peak expression levels during replicative trophozoite stage and schizont stage. A previous study from our laboratory has shown the upregulation of PfPCNA1 in the presence of genotoxic agents (Mitra, Banu et al. 2015) prompted us to further our studies related to investigate the role of PCNAs in DNA damage response. In *P. falciparum*, the components of Base Excision Repair (BER) pathway (Haltiwanger *et al*., 2000) and a putative Nucleotide Excision Repair (NER) pathway (Trotta *et al*., 2004) have been identified. Kirkman, *et al.* highlighted the importance of homology independent alternative end joining mechanism in *Plasmodium*, in addition to Rad51 mediated homologous recombination pathway (Roy *et al*., 2014) to repair double strand DNA breaks.

PCNA's role has been extensively studied in response to DNA damage in higher eukaryotes including mammalian cells and plants. Our results showing PfPCNA1 & 2

protein stability, chromatin enrichment, increment in PCNA1 foci numbers and PCNA2 enrichment in the nucleus following DNA damage accompanied with PfRad51 upregulation suggests for an activation of a repair pathway by mode of homologous recombination. PfRad51, PfRad54 and PfRPA (Replication protein A) were found to be key components of recombination machinery in the parasite (Bhattacharyya and Kumar 2003, Gopalakrishnan and Kumar 2013).

Chromatin stability of PfPCNAs following DNA damage may be an outcome of a slowly moving replication fork and assembly of PCNA rings at damaged sites for allowing repair proteins to assemble at damaged sites. Long patch BER pathway demonstrated in *P. falciparum* (Haltiwanger, Matsumoto et al. 2000) is a PCNA dependent process requiring an active FenI in later steps of DNA repair (Frosina, Fortini et al. 1996, Krokan, Nilsen et al. 2000). The functional transition of PCNA from soluble to chromatin bound form following DNA damage is considered to reflect the DNA repair activity mediated by Pol δ or Pol ε (Tsurimoto 1999). PCNA stability in *P. falciparum* may also be playing an important role in DNA damage response pathway by allowing repair re-synthesis to occur at damaged sites through LP-BER pathway.

We have found the upregulation of PfPCNAs at the protein level but not at the transcript level following DNA damage. It may occur due to the reduced turnover or stabilization of the existing protein in the parasite through possible post translational regulation.

However, the upregulation of PfRad51 following DNA damage is corroborated by an increase in the transcript level. These results suggest differential regulation of DNA damage inducible genes within the same parasite.

Role of PfPCNAs in damaged parasites was further demonstrated upon over-expression of both PfPCNA1 and PfPCNA2 that led to better survival ability of the parasites under the influence of genotoxic agents as compared to the control parasites. We believe that the parasite has evolved multiple pathways to deal with DNA damage where PfPCNAs play important roles. We propose a model where different roles of PfPCNAs in general DNA metabolism and in response to DNA damage (**Figure 1**) have been shown. Both the PfPCNAs may be required by the parasite to act on distinct DNA damage response

pathways to restore genomic stability, whereas PfPCNA1 may be involved in DNA replication primarily. Thus, a more robust DNA damage response pathway mediated by two distinct PCNAs could be the key to the success of the parasite survival in the presence of genotoxic agents.

PCNA undergoes several post-translational modifications (PTMs) including ubiquitination, sumoylation, acetylation and phosphorylation for its regulation in mammalian and yeast systems (Moldovan, Pfander et al. 2007). We have identified a conserved tyrosine residue in PfPCNA1, Y213 which suggests its importance in terms of chromatin stability of PfPCNA1 as reported in mammalian cells earlier (Wang, Nakajima et al. 2006). Probably, phosphorylation of this Y213 site leads to its association with the chromatin and a non phosphorylated form remains in the parasite cytosol. We were unable to show phosphorylation of Y213 residue under our experimental conditions and it is important to mention that phosphoproteome data of *P. falciparum* also failed to detect Y213 phosphorylation. However, the over-expression of the mutant Y213F PCNA1 failed to rescue the parasites effectively following DNA damage compared to the wild type PCNA1. These results suggest the importance of Y213 residue in PCNA1 function and highlights evolutionary conservation among eukaryotes.

We also investigated another phosphosite, S191 in PfPCNA1 detected in phosphoproteome data of schizont stage enriched *P. falciparum* parasites (Treeck, Sanders et al.). Phosphorylation at the Serine residue at 191 position of PfPCNA1 has been established by the present and previous study from our laboratory (Dhar S. K.) as evident from diminished phosphorylation in *in vitro* kinase assay and response of parasite lines over-expressing S191A PCNA1 in rescue experiments. S191A mutation and subsequent loss of phosphorylation at this residue did not result in a significant effect on functional loss of PfPCNA1 in overcoming the effects of DNA damage in parasite survival. These mutations (both Y213F and S191A) also did not lead to a structural loss of PCNA trimerisation.

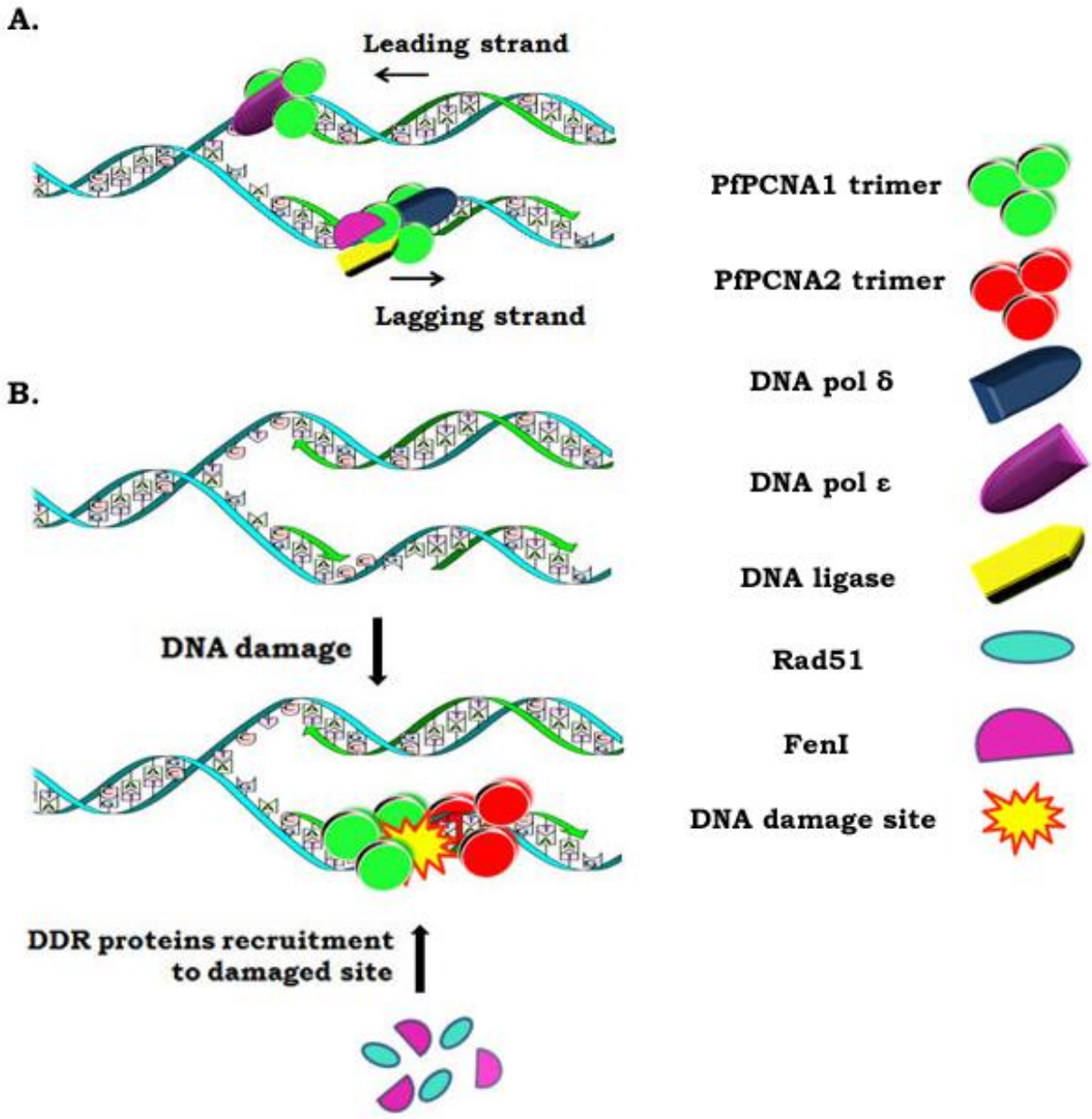


**Figure 1. Model depicting role of PfPCNA1 in DNA replication (A) and involvement of both PfPCNAs (PfPCNA1 and PfPCNA2) in DNA damage response. (A)** During DNA replication PfPCNA1 is required for leading strand synthesis as well as lagging strand DNA synthesis by interacting with respective DNA polymerases (epsilon and delta). Additionally, PfPCNA1 may interact with FEN1 and DNA ligase for lagging strand synthesis. **(B)** In response to DNA damage, both PfPCNA trimers (1 and 2, respectively) assemble at the site of damage along with other DNA damage response proteins including Rad51.

Therefore, a change in functional activity of PfPCNA1 could be the lack of phosphorylation at these sites for Y213F mutation, but it would be irrelevant to comment on the role of S191 at this stage based on our findings.

Presence of PfPCNAs in the cytoplasmic fraction as well as in the nucleus suggests that PCNA plays a diverse range of functions. Functions associated to nucleic acid metabolism is assigned to chromatin associated form of PCNA, however other metabolic functions and prevention of apoptosis may be fulfilled by cytoplasmic pool of PCNA (Naryzhny and Lee 2010). The cytoplasmic pool of PCNA may shuttle on and off the nuclear DNA as and when required by the cell.

Diverse functions of PfPCNAs in *P. falciparum*, various associated modifications and interplay of PfPCNA1 and PfPCNA2 may be investigated further. Any strategy which may target these molecules could potentially affect parasite progression. Our present understanding of PCNA-dependent mechanisms is still very limited in *P. falciparum*, and a *pcna1* and *pcna2* knockout analysis is advisable and under progress to study in detail the functional relevance of these two genes.

# LIST OF PUBLICATIONS

# Functional dissection of proliferating-cell nuclear antigens (1 and 2) in human malarial parasite *Plasmodium falciparum:* possible involvement in DNA replication and DNA damage response

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Eukaryotic PCNAs (proliferating-cell nuclear antigens) play diverse roles in nucleic acid metabolism in addition to DNA replication. *Plasmodium falciparum*, which causes human malaria, harbours two PCNA homologues: PfPCNA1 and PfPCNA2. The functional role of two distinct PCNAs in the parasite still eludes us. In the present study, we show that, whereas both PfPCNAs share structural and biochemical properties, only PfPCNA1 functionally complements the ScPCNA mutant and forms distinct replication foci in the parasite, which PfPCNA2 fails to do. Although PfPCNA1 appears to be the primary replicative PCNA, both PfPCNA1 and PfPCNA2 participate in an active DDR (DNA-damage-response) pathway with significant accumulation in the parasite upon DNA damage induction. Interestingly, PfPCNA genes were found to be regulated not at the transcription level, but presumably at the protein stability level upon DNA damage. Such regulation of PCNA has not been shown in eukaryotes before. Moreover, overexpression of PfPCNA1 and PfPCNA2 in the parasite confers a survival edge on the parasite in a genotoxic environment. This is the first evidence of a PfPCNA-mediated DDR in the parasite and gives new insights and rationale for the presence of two PCNAs as a parasite survival strategy and its probable success.

Key words: DNA-damage response, DNA replication, PCNA, *Plasmodium falciparum*, replication foci.

## INTRODUCTION

PCNA (proliferating-cell nuclear antigen) was discovered by Miyachi et al. [1] as an antigen recognized by certain autoantibodies from autoimmune disease systemic lupus erythaematosus patients. Later PCNA was demonstrated to be the DNA polymerase auxiliary factor [2,3]. It has become evident over the last two decades that PCNA is essential not only for DNA replication, but also for other aspects of nucleic acid metabolism, including DNA repair, translesion DNA synthesis, post-replicative cytosine methylation, chromatin assembly and overall cell cycle progression [4,5]. This multifunctional nature of PCNA protein is facilitated because of its ability to interact with wide array of proteins [6,7].

A short consensus sequence Q-XX-(h)-X-X-(a)-(a), where 'h' represents residues with moderately hydrophobic side chains (e.g. leucine, isoleucine or methionine), and 'a' represents residues with highly hydrophobic aromatic side chains (e.g. phenylalanine or tyrosine), termed the PIP (PCNA-interacting protein) motif, has been identified in numerous proteins and demonstrated to facilitate a conserved mode of interaction with PCNA [8,9]. Structural studies have shown that the specificity for different interacting proteins may arise from subtle conformational differences in the hydrophobic pocket on the surface of different PCNAs, enabling them to discriminate between PIP motifs of differing structure and sequence [10]. A study in yeast has demonstrated that PIP motifs are present in all the three component subunits of DNA Pol (polymerase) $\delta$ and each of them contributes to PCNA-stimulated DNA synthesis by Pol $\delta$, and mutational inactivation of all three PIP motifs abrogates its ability to synthesize DNA with PCNA [11]. Enzymes central to Okazaki fragment maturation, FEN-1 (flap endonuclease 1) and DNA ligase I have been shown to interact with PCNA through their PIP motif [12,13] with a probable cyclical recruitment of the enzymes on the PCNA platform [14].

In general, there is only one gene encoding PCNA in eukaryotes, but there certainly are exceptions. Several members of the Archaea, a number of plants and Apicomplexan parasites including *Toxoplasma gondii* and *Plasmodium falciparum* have been found to contain more than one PCNA homologue [15–20]. In the archaeon *Thermococcus kodakaraensis*, the two PCNA (TkPCNA) proteins support processive DNA synthesis by the polymerase. Both proteins form trimeric structures with characteristics similar to those of other Archeal and Eukaryal PCNA proteins. One of the notable differences between the TkPCNA rings is that the interfaces are different, resulting in different stabilities for the two trimers [21]. *Arabidopsis thaliana* also possesses two genes for PCNA. Using *in vitro* and *in vivo* approaches, it was observed that PCNA2, but not PCNA1, physically interacts with DNA Pol $\lambda$, enhancing its fidelity and efficiency in translesion synthesis [22]. Both AtPCNAs form homotrimeric ring structures which are essentially identical with

Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; DDR, DNA-damage response; DSB, double-strand break; FEN-1, flap endonuclease 1; FOA, 5-fluoro-orotic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HU, hydroxyurea; IFA, immunofluorescence assay; MMS, methyl methanesulfonate; ORC, origin recognition complex subunit; ORC1C, C-terminal domain of ORC1; ORC1N, 238 amino acid residues from the N-terminus of ORC1; PCNA, proliferating-cell nuclear antigen; Pf, *Plasmodium falciparum*; PIP, PCNA-interacting protein; Pol, polymerase; qPCR, quantitative real-time PCR; RT, reverse transcription; ScPCNA, *Saccharomyces cerevisiae* PCNA; TgPCNA, *Toxoplasma gondii* PCNA; TkPCNA, *Thermococcus kodakaraensis* PCNA; YPD, yeast extract/peptone/dextrose.

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*Published online 8 September 2015* *Nucleic Acids Research, 2015, Vol. 43, No. 18* ***8651–8663***
*doi: 10.1093/nar/gkv875*

# Sequence-specific recognition of DNA minor groove by an NIR-fluorescence switch-on probe and its potential applications

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Received June 26, 2015; Revised August 12, 2015; Accepted August 19, 2015

## ABSTRACT

**In molecular biology, understanding the functional and structural aspects of DNA requires sequence-specific DNA binding probes. Especially, sequence-specific fluorescence probes offer the advantage of real-time monitoring of the conformational and structural reorganization of DNA in living cells. Herein, we designed a new class of D2A (one-donor-two-acceptor) near-infrared (NIR) fluorescence switch-on probe named quinone cyanine–dithiazole (QCy–DT) based on the distinctive internal charge transfer (ICT) process for minor groove recognition of AT-rich DNA. Interestingly, QCy–DT exhibited strong NIR-fluorescence enhancement in the presence of AT-rich DNA compared to GC-rich and single-stranded DNAs. We show sequence-specific minor groove recognition of QCy–DT for DNA containing 5′-AATT-3′ sequence over other variable (A/T)4 sequences and local nucleobase variation study around the 5′-*X*(AATT)*Y*-3′ recognition sequence revealed that *X* = A and *Y* = T are the most preferable nucleobases. The live cell imaging studies confirmed mammalian cell permeability, low-toxicity and selective staining capacity of nuclear DNA without requiring RNase treatment. Further, *Plasmodium falciparum* with an AT-rich genome showed specific uptake with a reasonably low $IC_{50}$ value (<4 μM). The ease of synthesis, large Stokes shift, sequence-specific DNA minor groove recognition with switch-on NIR-fluorescence, photostability and parasite staining with low $IC_{50}$ make QCy–DT a potential and commercially viable DNA probe.**

## INTRODUCTION

Sequence-specific recognition is an essential criterion to target and control DNA functions for gene-expression, bioimaging, diagnostics, therapeutics and biotechnological applications (1,2). Over the years, many probes have been developed to target DNA, but there is still a pressing need for developing efficient new probes and therapeutic agents against gene-related diseases as DNA remains a promising biological receptor (3–6). It is a daunting task indeed to design site-specific DNA binding molecules with high affinity and selectivity. To achieve this goal, base-pair and sequence-selective DNA binding probes ranging from small molecules to large peptides of natural and synthetic origin have been developed (7–11). These probes interact with DNA mainly through two binding modes, intercalation and groove binding. Typically, small molecules binding to DNA through intercalation possess the site-specificity of three base pairs that can differentiate only one out of 32 random sequences (12). Factually, the human genome contains 3 billion base pairs and the small molecular probe is posed with the astonishingly large number of 1 billion unique binding sites (13). To improve the binding specificity of small molecular probes over longer DNA sequences, researchers shifted their attention toward groove-binding agents. DNA grooves are sites with inherent hydrogen bond donors and accep-

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