BIOCHEMICAL CHARACTERIZATION OF MGS1 IN *SACCHAROMYCES CEREVISIAE*

by

Aditi Khankhoje

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Chemistry and Biochemistry

Summer 2015

BIOCHEMICAL CHARACTERIZATION OF MGS1 IN *SACCHAROMYCES CEREVISIAE*

by

Aditi Khankhoje

Approved: _____
Zhihao Zhuang, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Murray Johnston, Ph.D.
Chair of the Department of Chemistry and Biochemistry

Approved: _____
George H. Watson, Ph.D
Dean of the College of Arts and Sciences

Approved: _____
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would like to sincerely thank Dr. Zhihao Zhuang for accepting me as his student and guiding me throughout my graduate studies at University of Delaware. Without his persistent efforts and rigorous mentoring this thesis would not have been possible. It is he who has instilled in me the attitude of hard work and focused efforts towards a specific goal. He taught me the importance of reading to cultivate scientific thought and try to be ahead of my peers in the field. There were times when I felt lost and defeated, but he was always there to reassure and motivate. He assured a very professional environment in the lab and that helped me appreciate the value of being organized and being up to date. He taught me the importance of designing good controls for experiments, which is a very important factor of research and is seldom appreciated by new graduate students.

I would also like to thank Kun Yang, who mentored me through my first year and helped me get started in the lab. He has been crucial in my development as a Biochemist. I will always regard him as an exceptional scientist and an even better human being. I wish him luck in his life and career. I would also like to thank my current and past group members Qin Liang, Adam Tencer, Jason Cargill, Guorui Li, Ping Gong, Christine Ott, Rosemary Flores, Siqi Shen, Weijun Gui, Libo Yuan and Prajwal Paudel for always being there in thick and thin.

To my parents, I owe my greatest gratitude-Aai and Baba-Dr. Sanjeevani Khankhoje and Milind Khankhoje have been standing firm besides me in all my life decisions and endeavors. Aai has been the greatest force behind me and I can't thank her more for making this world so beautiful for me. I can't forget her undiminished dedication towards her work as a doctor and all those tough times when she stood against all odds to bring me up. If people choose to call me a well brought up human being today, it is due to her sustained efforts.

I would also like to thank my brother Akshay for being there for me, always. Can't forget the day I saw him for the first time. He is and will always be the most precious person to me.

Also I would also like to thank my god parents and in-laws, Sujata Joglekar and Dileep Joglekar, who accepted me graciously into their family. They never doubted my abilities and always helped me look ahead in toughest of times. It is because of their unequivocal support and trust that I could balance my research and responsibilities towards our family. I am lucky to have them in my life.

Last but certainly not the least, I would like to thank my husband Pushkar, who has been with me through all those times of happiness and helplessness and whom I have grown to love. It is he who held the 5 years long, long-distance relationship together. For him, till death and beyond.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Throughout the life cycle of a cell, the DNA is damaged as a result of environmental and endogenous factors. Such damage causes aberrant changes in DNA topology which can lead to replication fork stalling and result in erroneous duplication. If stalled replication forks are left unresolved, double strand Breaks (DSB's) would form, causing random recombination events contributing to genome-wide instability. Mutations may accumulate over generations leading to a predisposition to cancer and other genetic diseases. Prokaryotes and eukaryotes have evolved robust DNA damage response mechanisms to overcome genome instability caused by replication fork collapse. Proteins in these biochemical pathways are highly conserved and garner a robust response to replication fork stalling events. It is thus conceivable that deficiencies in these proteins cause genetic disorders, making it imperative that these be studied and characterized in detail.

Mgs1, Maintenance of Genome stability 1, a member of the AAA^+ ATPase family is thought to be involved in the fork restart DNA damage response (DDR) pathway activated by replication stress in presence or absence of genotoxic agents. Previous published studies have shown that Mgs1 physically associates with poly-ubiquitinated PCNA and POL31 subunit of Polymerase δ. Genetic profiling has indicated interactions with the *RAD6* epistasis group. It has also been shown that,

although deficiency of Mgs1 in not lethal to the cell, it leads to increase in aberrant recombination and mutation. Interactions of Mgs1 with the enzymes involved in both replication and replication repair pathways makes it an interesting protein from a biochemical standpoint, shedding light on its possible role in the maintenance of genomic stability during and after replication.

Plethora of data exists, studying Mgs1 under various genetic backgrounds especially in that of replication stress. Hitherto, detailed biochemical analysis of DNA binding and ATPase activity of Mgs1 is not available. Here, we try to understand the complex nature of ATPase activity and the influence of different DNA structure-substrates on stimulating the activity. We also confirm the ATPase activity by creating a null ATPase mutant; K183A. We study the DNA binding of Mgs1 and its regulation by ATP analogs.

Our data suggests clear difference in stimulation of ATPase activity in the presence of single stranded DNA and double stranded DNA structure. Also we show here that the DNA binding affinities are dictated by the DNA structure and ATP influences this activity.

Our findings help us delineate a mechanistic model for the interaction of Mgs1 with DNA and to place these activities in the perspective of the available genetic data for Mgs1.

Chapter 1

INTRODUCTION

1.1 DNA Replication

DNA has been described as a "Cell's Master Molecule"[1] and is central to the survival, growth, and replication of the cell. The encrypted code in the DNA has to be deciphered and executed upon for cell cycle progression and cell division. The process for duplication of DNA is called replication, in which the DNA is copied from the template to a daughter DNA which in turn is transferred to the daughter cell upon cell division.

Mitosis is a process in which chromosomes replicate to give rise to identical chromosomes. Two sets of chromosomes so formed are enclosed in the nuclear envelop and following cytokinesis separate into two daughter cells. Proper replication of DNA, thus, holds the key to sustain generations of organisms and therefore is the most important event for species propagation.

DNA replication takes place in the S-phase between G1 and G2 phases and is tightly regulated by check-point regulations[2]. As many as 60 proteins are involved in the process of replication. Eukaryotic replication is initiated by a multimeric protein complex called origin recognition complex (ORC)[3]. In eukaryotes many origins fire at the same time in order to maintain the rate of replication. Firing of origin of

replication has been recently studied in-vitro reconstituted system[4]. The initiation of replication is divided into two brief stages in which the MCM complex is loaded on origin in the G1 phase and is converted into functional CGM complex in S phase. This event leads to the replication start at different origins.

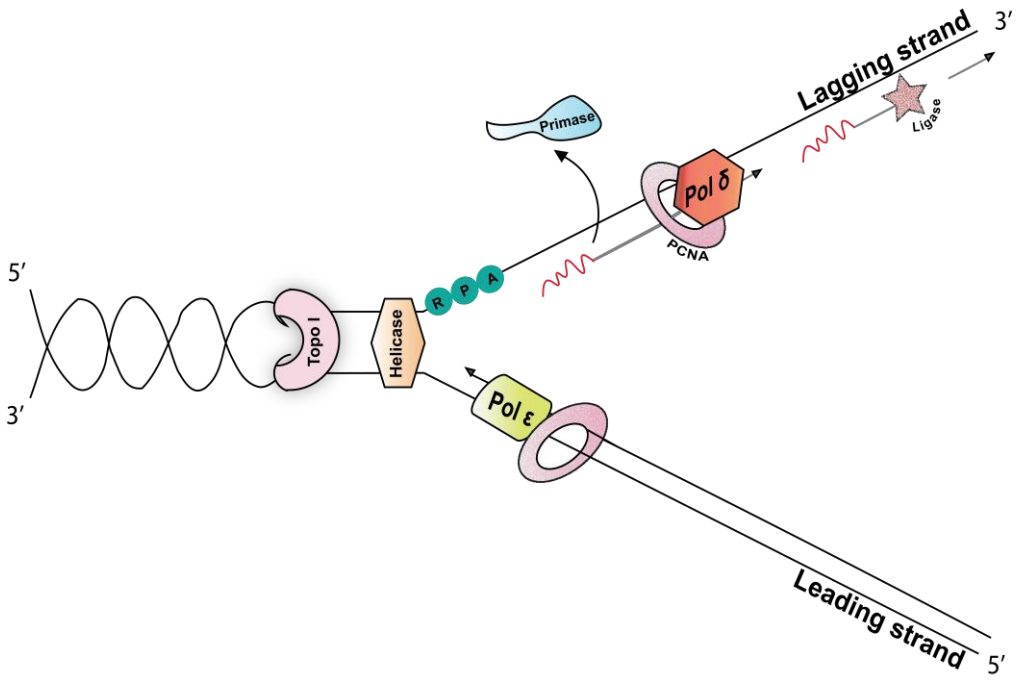


Figure 1.1: **DNA replication fork**. The 'replication fork' structure formed as a result of advancing replication on the leading and the lagging strand.

The replication is initiated on both strands of the template DNA and is coupled to maintain a steady rate[5]. Leading strand is the DNA strand which is replicated in the direction following the helicase. Replisome is involved in continuous replication on

the leading strand in 5'-3' direction. The replication of the leading strand is mediated through the polymerase ε during undisturbed replication[6].

The replication on the lagging strand is discontinuous. Replicative polymerases possess polarity and can synthesize DNA in 5'-3' direction. This causes the lagging strand to be synthesized in discrete short fragments. Primase synthesizes short stretches of RNA "Primers". DNA polymerase extends the RNA primers in the 5'-3' direction, these short DNA segments are called Okazaki fragments. Polymerase δ replicates the DNA on the lagging strand.

One of the most primal aspects for the continuity of life is maintaining the integrity of the genome. Therefore, the maintenance of fidelity with each round of replication is crucial. In this regard, the DNA polymerase replicate with proof reading abilities. These proof reading abilities are granted by 3'-5' exonuclease activity of DNA polymerases[7,8]. The error rates are as low as 10^{-9} to 10^{-11} for replicative DNA polymerases in *E.coli*[9].

Impedance of replication fork by environmental or endogenous factors causes stalling[10]. Replication fork stalling can lead to genome instability and is a source of serious threat of mutation accumulation, which can lead to predisposition for cancer.

1.2 Replication Fork Stalling

A detailed account of the various endogenous and exogenous sources of DNA damage is summarized in a recent review by Nicola J. Curtin[11]. Figure 1.2 adopted from the paper, develops a landscape of the possible of DNA damaging agent. It also

illustrates “the approximate number of the indicated type of lesion that occurs naturally in a cell each day”. The cell is evidently overwhelmed by DNA damage, nevertheless it is heavily armored with specialized repair pathways to deal with it. The repair mechanisms are tightly regulated but interchangeable such that the impairment caused by loss of mediator of one pathway is taken up by another repair pathway.

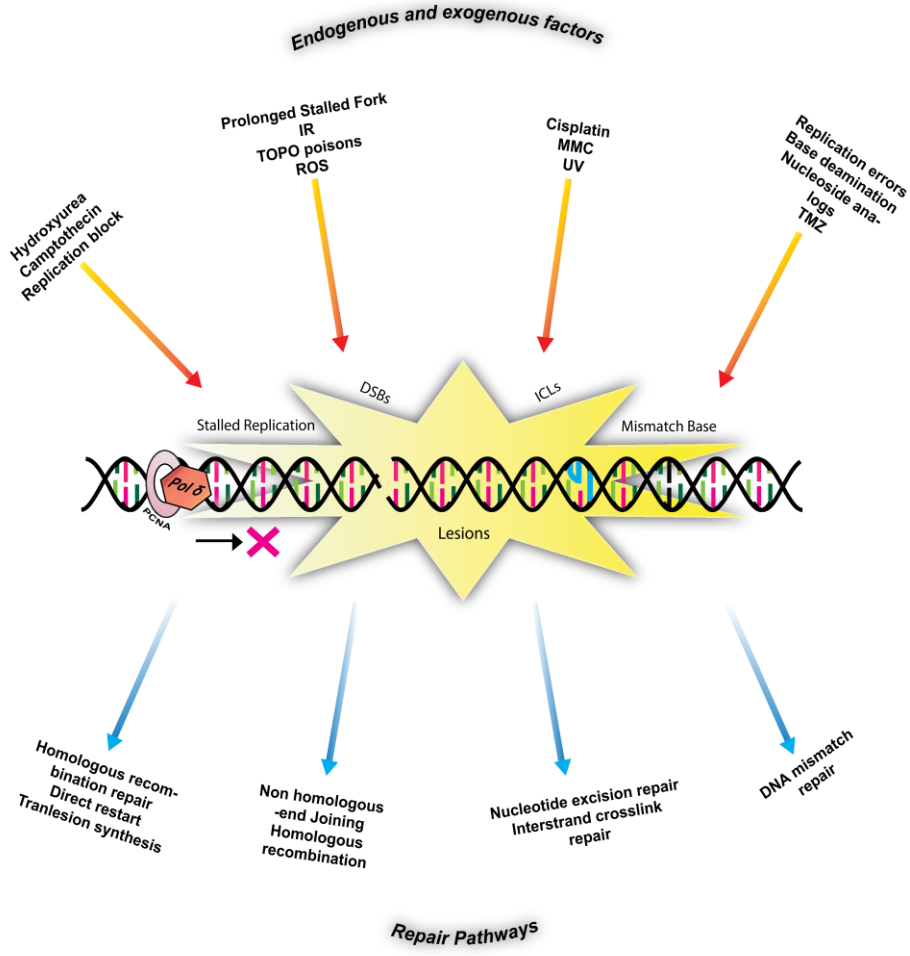


Figure 1.2: **Sources of DNA Damage and the Associated Repair Mechanism.**

Replication fork stalling could result from encountering tightly bound Proteins, altered dNTP concentration, bulky adducts causing physical stalling of replisome or secondary structures of the DNA. Chemical agents like *hydroxurea* and *aphidicolin* also cause replication fork stalling or collapse. Considering a high frequency of these types of replication stalling agents, robust mechanism of 'fork restart' is available in form of repair proteins and checkpoints[12]. Cell cycle progression is regulated by these checkpoints.

The pathways in the fork stalling and collapse in mammalian cells has been extensively discussed by Eva Petermann and Thomas Halleday[13]. Stalled replication in eukaryotes is especially difficult to understand because of the presence of multiple repair mechanisms and their interplay. Replication can be resumed by different mechanisms depending upon the type of replication fork block and the time DNA is subjected to it.

The time of the replication fork block is also crucial in order for a particular replication restart pathway to be activated as illustrated in Figure 1.3. Following a short exposure of 2-4 hours, the fork is directly restarted. It is possible that there are accessory replication proteins involved in stabilizing the fork during such short blocks. Prolonged blocks cause a complete collapse but can be resumed after MUS81 mediated formation of DSBs which can be resolved by recombination mediated restart pathways.

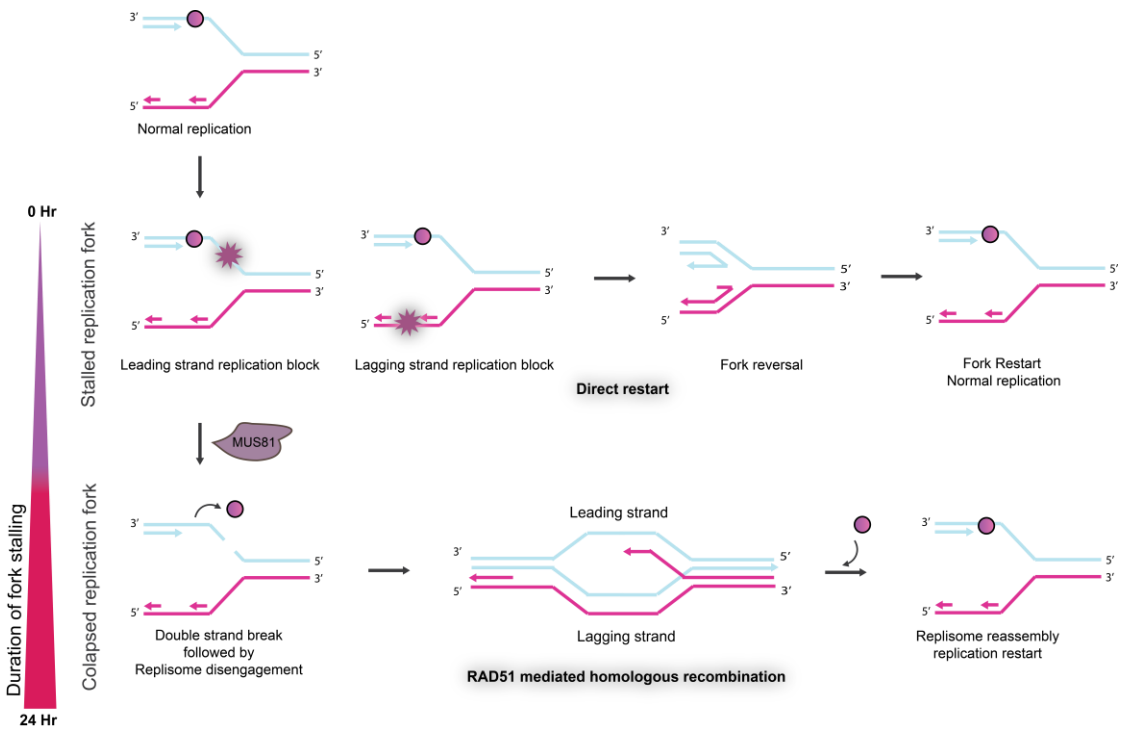


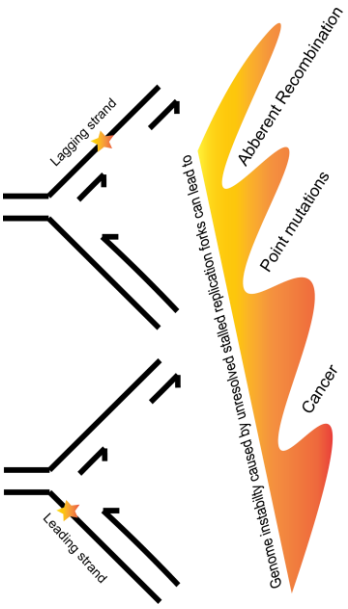
Figure 1.3: **Effect of time of chemical replication block on the resolution of the stalled replication fork.** Replication associated DNA damage repair proteins mediate a direct restart if the replication block causing agent (hydroxyurea) is removed early. Prolonged block causes the inactivation of the forks. Following which, MUS81 mediated DSBs are formed. DSB promote recombination mediated repair and firing from new origins.

1.3 AAA+ ATPase Family

AAA+ ATPase super family gets its name from the highly conserved core AAA+ module[14,15], involved in binding and hydrolysis of ATP. The module consists of αβα core domain which harbors the Walker A and Walker B motifs, as described in 1982 by John Walker[16]. The β- and γ- phosphates of ATP bind together with Mg^{2+} cation on the conserved lysine and serine/threonine residues of the Walker A motif. The Walker B motif is involved in the hydrolysis of ATP[17].

AAA+ ATPases have diverse function and oligomeric structures. A detailed account of AAA+ ATPases is discussed by Teru Ogura and Anthony J. Wilkinson[14]. In the review, they attempt to describe the plethora of proteins in this super family and associated diverse function and structure.

1.4 Motivation



Mgs1 remains to be a very poorly understood even though vast amount of genetic data exists. As recent as 2012, Helle D. Ulrich, in the research paper, presents many unresolved biochemical questions associated with Mgs1[18]. Curiously, Mgs1 has Ubiquitin interacting 'UBZ domain' and also DNA mediated activities. It is of profound interest of how these activities influence the physiological role of Mgs1. Although, Mgs1 does not appear to be an important factor for survival of the cell, its absence causes elevated rates of recombination which suggests a potential role in maintaining genome stability. Proteins involved in maintaining genome stability are of keen scientific interest because of the cancer causing mutations accumulating in the cell in their absence.

There is also a striking functional similarity between stalled replication fork repair proteins SMARCAL1 and ZRANB3 that needs to be addressed.

Chapter 2

RESULTS

2.1 Expression and Purification of Mgs1 (wt) and Mgs1 (K183A)

Mgs1 (wt) and Mgs1 (K183A) were cloned in pET21a vector, expressed and purified from Rosetta DE3 cells. Mgs1 (wt) and (K183A) was purified using nickel affinity chromatography. The representative SDS-Page purification gels are shown in the (Figure 2.2 A). The DNA stimulated ATPase activity was observed for Mgs1 (wt) as reported earlier [19]. The ATPase mutant (K183A) was confirmed by the loss of ATPase activity in the NADH enzyme-linked ATPase assay (Figure 2.2 B).

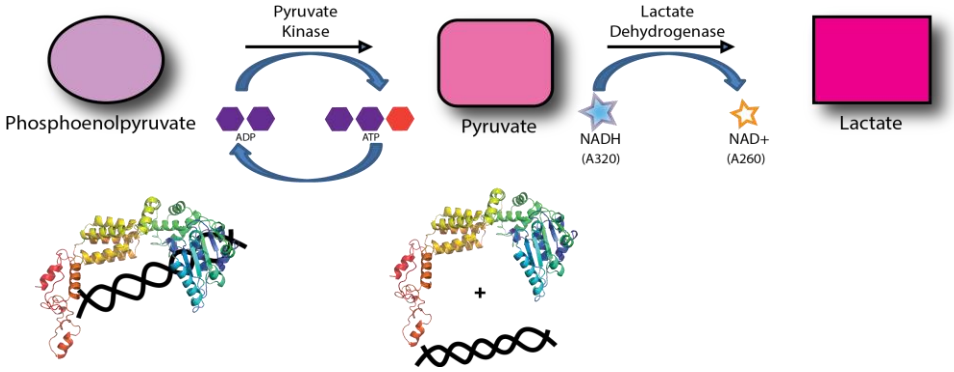


Figure 2.1: **NADH Enzyme-linked ATPase assay**. A Schematic representation of NADH Enzyme-linked ATPase assay. ATP which is consumed by ATPase domain of Mgs1 (wt), is regenerated from ADP by Pyruvate kinase which thereby converts phosphoenolpyruvate to pyruvate. Lactate dehydrogenase sequentially converts Pyruvate to Lactate utilizing NADH. Oxidation of NADH to NAD+ can be monitored at 340nm.

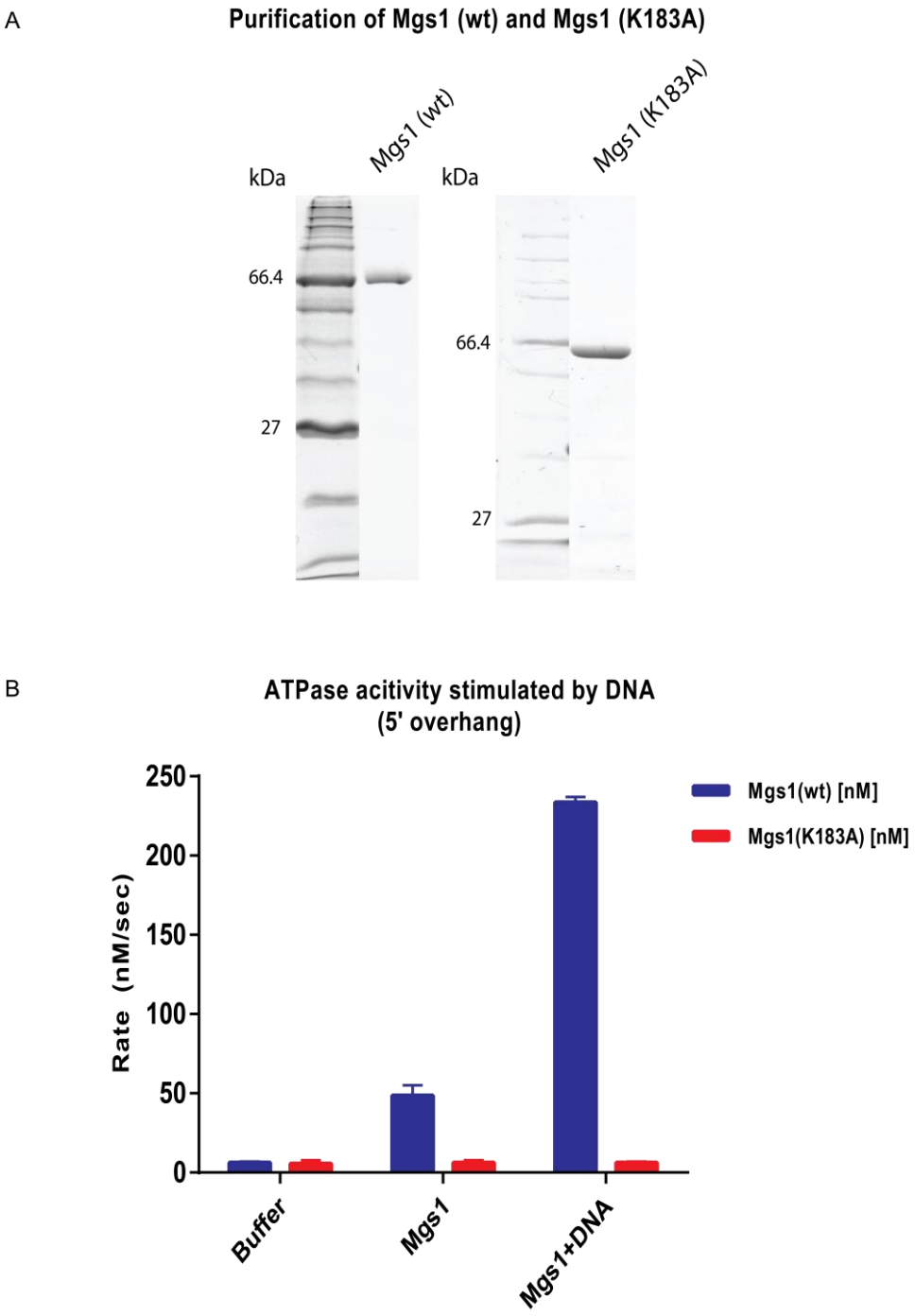


Figure 2.2: **Mgs1 (wt) and Mgs1 (K183A) Purity and activity**. (A) SDS-PAGE analysis of full length Mgs1 (wt) and Mgs1 (K183A) (B) Rate of ATPase activity for the Mgs1 (wt) and Mgs1 (K183A)

2.2 DNA Structure Stimulated ATPase Activity

Mgs1 is proposed to modulate DNA topology[19], also is has been shown that Mgs1 stimulates activity of Fen1, but does not physically interact with Fen1 as confirmed by immuno-pull down assay[20]. The modulation of Fen1 is likely due to Mgs1's interaction with DNA rather than Fen1. The catalytic ATPase domain might be involved in these activities. Studies suggest that many proteins of this type possess DNA structure specific ATPase activity[21]. Mgs1 has been shown to have DNA-structure specific changes in ATPase simulation[20]. In order to understand the effect of DNA structure on ATPase of Mgs1, ATPase assay was performed. Six different DNA-Structure substrates were designed for a NADH Enzyme-linked ATPase assay. The initial velocities were determined at various DNA Structure substrate concentrations that ranged from 50 nM to 1 μM in presence of 1mM ATP. The data was fit to the Michaelis-Menten equation to obtain *k*cat and *K*m. The data suggests no significant change in the kinetic profile of Mgs1 in presence of DNA-structure templates as demonstrated in (Table 2.1). But it is interesting to note that the rate of ATP hydrolysis changes significantly for different DNA structures.

These can be grouped into three categories. First, DNA structures with single stranded character, second, DNA structures with double stranded character and third, DNA structures with both single and double stranded character. Mgs1 ATPase activity is highly stimulated by DNA structure having both single and double stranded character and least by Single stranded DNA (Figure 2.4).

Table 2.1: Steady-state kinetic properties of wild type Mgs1

	DNA structure	K_m (nM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
1	**19nt Oligo**	53.09	0.146	$2.75x10^6$
2	**40nt Oligo**	52.37	0.154	$2.94x10^6$
3	**Blunt**	19.18	0.205	$10.6x10^6$
4	**5' Overhang**	54.55	0.275	$5.06x10^6$
5	**3' Overhang**	49.13	0.280	$5.69x10^6$
6	**Fork**	33.90	0.263	$7.75x10^6$

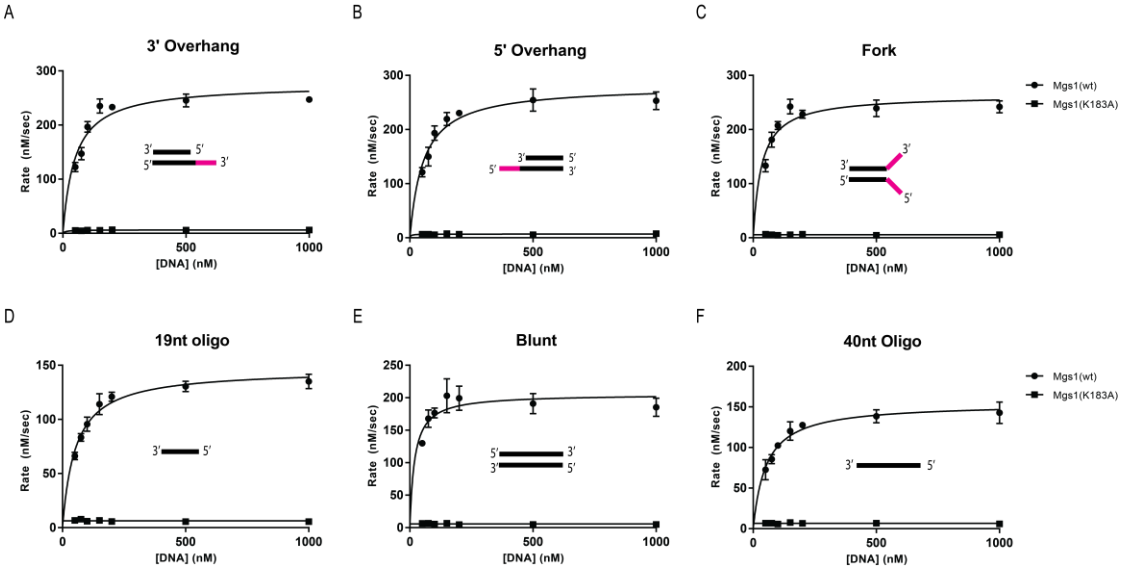
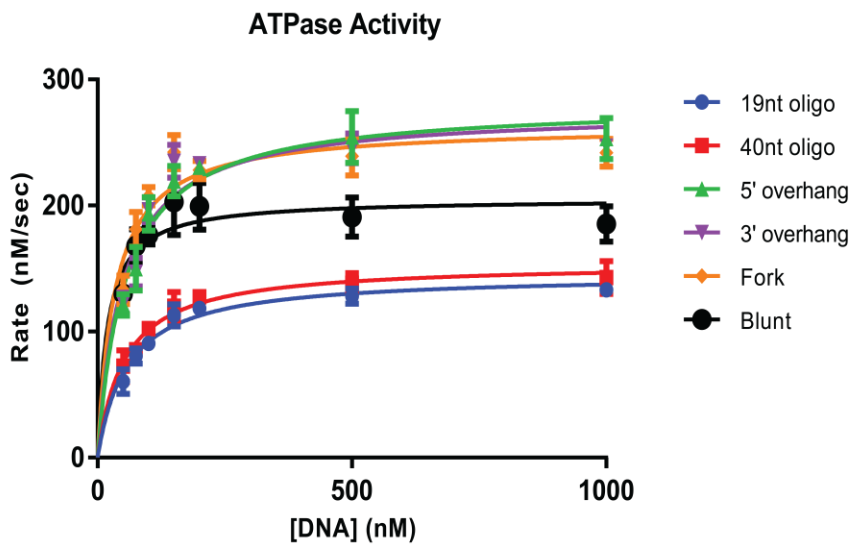


Figure 2.3: **Individual rates of ATP hydrolysis by Mgs1 in presence of DNA-Structures**. (A) Rate for 3' Overhang. (B) Rate for 5' Overhang. (C) Rate for Fork. (D) Rate for 19nt long oligonucleotide. (E) Rate for Blunt ended oligonucleotide. (F) Rate for 40nt long oligonucleotide.

A



B

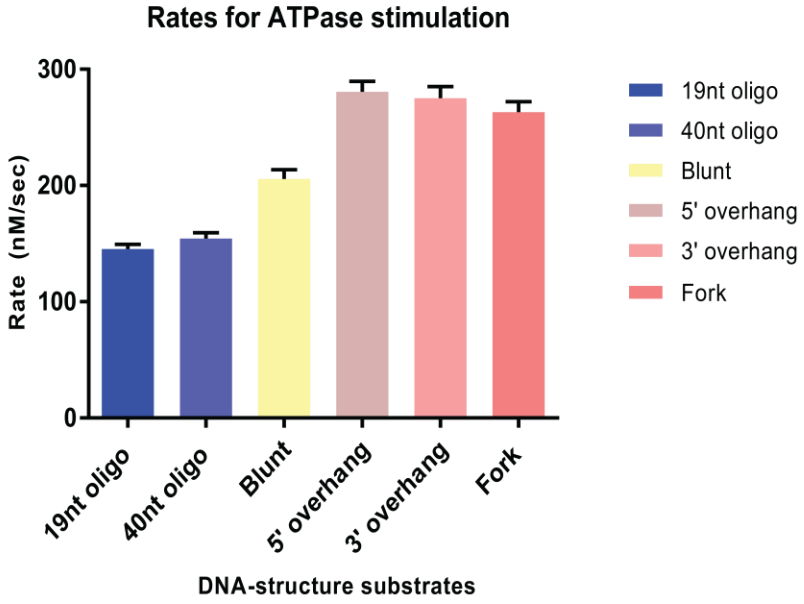


Figure 2.4: **Effect of DNA-structure templates on rate of ATP hydrolysis by Mgs1.** (A) and (B) The rate of ATP hydrolysis was recorded in the range of 50nM-1uM of the DNA concentration and 1uM of Mgs1 concentration.

2.3 Optimization of Fluorescence Polarization Assay for DNA Binding Assay

In order to get an insight into DNA binding of Mgs1, a fluorescence polarization (FP) based assay was used. Optimization of the FP assay was performed by studying the DNA binding of RPA (Replication protein A), a single stranded DNA binding protein homologous to bacterial SSB (Single Stranded Binding protein).

3'end 6-FAM (Fluorescein) labelled DNA substrate was used in this assay. The scheme is shown in the Figure 2.5.

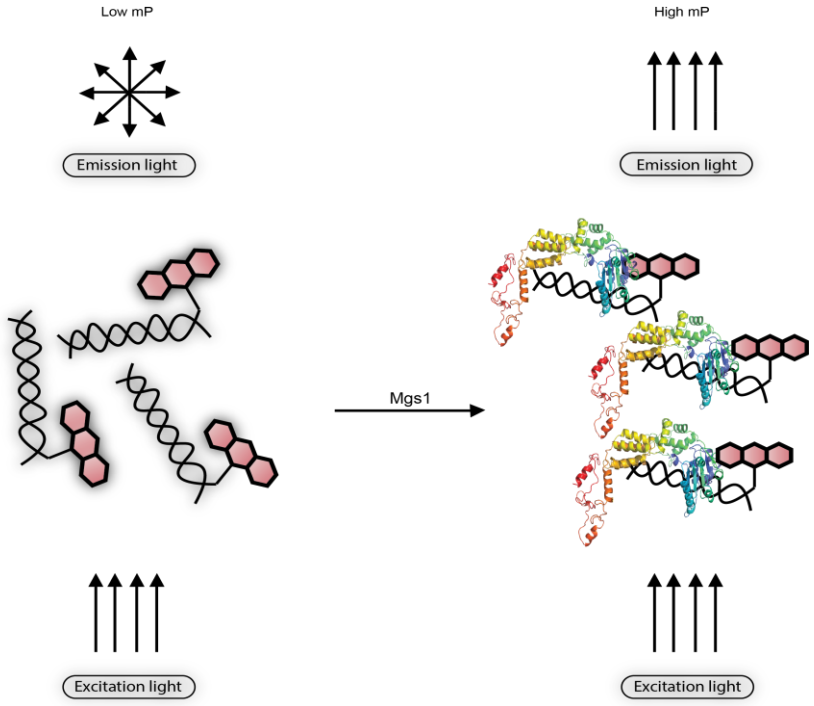


Figure 2.5: **Principle of fluorescence polarization**. The 6-FAM Fluorophore modified oligonucleotide is excited by plane polarized light obtained by passing through an excitation polarizing filter; the polarized fluorescence is measured through an emission polarizer either parallel or perpendicular to the exciting light's plane of polarization. Two intensity measurements are obtained (I⊥ and I||) and used for the calculation of FP.

The ⊥ and || Intensities obtained at emission filter were used to calculate the fluorescence polarization (mP) values.

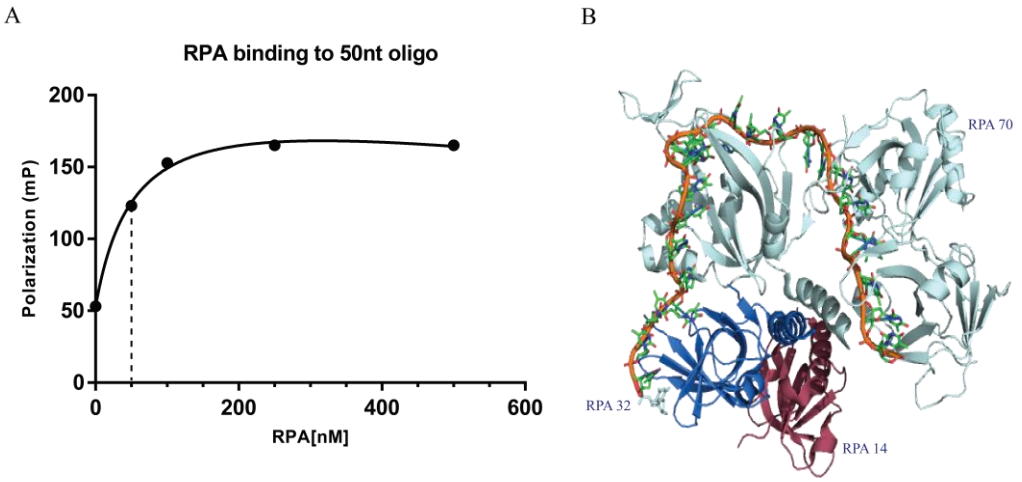


Figure 2.6: **RPA binding to ssDNA**. (A) Binding curve of RPA-ssDNA binding by Fluorescence polarization. (B) Co-crystal structure of 25nt ss-DNA and RPA (PDB ID: 4GOP).

The co-crystal structure of RPA heterotrimer and single stranded DNA is available[22]. One RPA heterotrimer binds to 25 nucleotide stretch of ss-DNA (Figure 2.6 B). The binding assay was performed in presence of 100nM 3'end 6-FAM labelled 50nt oligonucleotide. A dissociation constant was subsequently obtained ($K_{D=}$55.19 nM) using the equation of the one site-total analysis in Graphpad Prism. The non-cooperative binding is confirmed from our studies as described earlier[23].

A qualitative competitive binding assay was performed to ascertain binding of Mgs1 to the target. Saturation binding was achieved at higher concentration on Mgs1, which was competitively inhibited by increasing concentrations of unlabeled DNA. The curve was fitted to the standard sigmoidal equation in Graphpad Prism (Figure

2.7). The binding was successfully inhibited, an indication of on target binding of Mgs1.

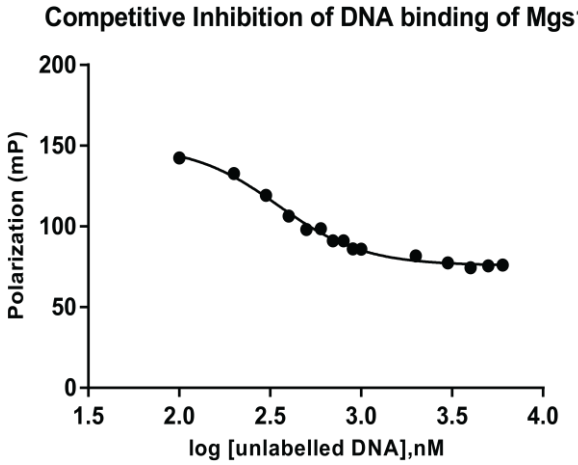


Figure 2.7: **Competitive inhibition of DNA binding by Mgs1**: Qualitative competition binding assay contained 1.4 uM Mgs1 and 100 nM labeled 50nt oligonucleotide, unlabeled 50nt oligonucleotide was titrated over a range of

2.4 Fluorescence Polarization for Understanding DNA Binding Specificities of Mgs1

DNA binding specificities may dictate physiological role of a protein in vivo. SMARCAL1, a DNA remodeling protein fundamental to genome stability during human cell replication, has been shown to demonstrate higher binding for fork structure than holiday junction[21] advancing the notion that SMARCAL1 acts on the stalled replication fork before it can be resolved into holiday junctions. RecG is a helicase in *Escherichia coli* involved in DNA recombination and repair, which in contrast to SMARCAL1, has been postulated to promote replication restart by

promoting the formation of Holliday junctions from stalled replication fork. It has been shown to have a binding preference for fork containing lagging strand rather than fork containing leading strand[24]. Also, Mgs1 has shown varied preferences in an electrophoretic mobility shift assay[20]. In order to have a more comprehensive understanding of DNA binding of Mgs1 we performed a fluorescence polarization DNA binding assay. DNA Structures were created by annealing 3'end 6-FAM labelled oligonucleotide with complementary unlabeled oligonucleotides as shown in Table 1.4. The DNA concentration was maintained at 100nM, whereas Mgs1 concentration was in the range of 0uM-2uM. Equilibrium binding data was plotted and fit to the one site-specific binding with hill slope, also accounting for the background in Graphpad prism. Each data point represents mean for repeats of three independent experiments.

We performed the DNA binding studies with simple structures gradually increasing the complexities of structures. "Simple structures" consisted of 5' Overhang, 3' Overhang, Fork, 50 nt oligonucleotide and Blunt ended DNA. The K_d for each structure is reported in the (Table 2.2).

Table 2.2: Dissociation constants for DNA binding of Mgs1 (Simple structures)

	DNA structure	K_d **(nM)**
1	**3' Overhang**	399±19
2	**50nt oligonucleotide**	436±26
3	**5' Overhang**	641±41
4	**Fork**	809±54
5	**Blunt**	881±14

As the results indicate Mgs1 shows a binding preference for 3’ overhang. This structure is predominantly found at the stalled replication fork on the lagging strand. To access whether this binding preference extends to a more complex DNA structure baring the 3’ overhang signature, further binding studies where performed using more complex DNA structures.

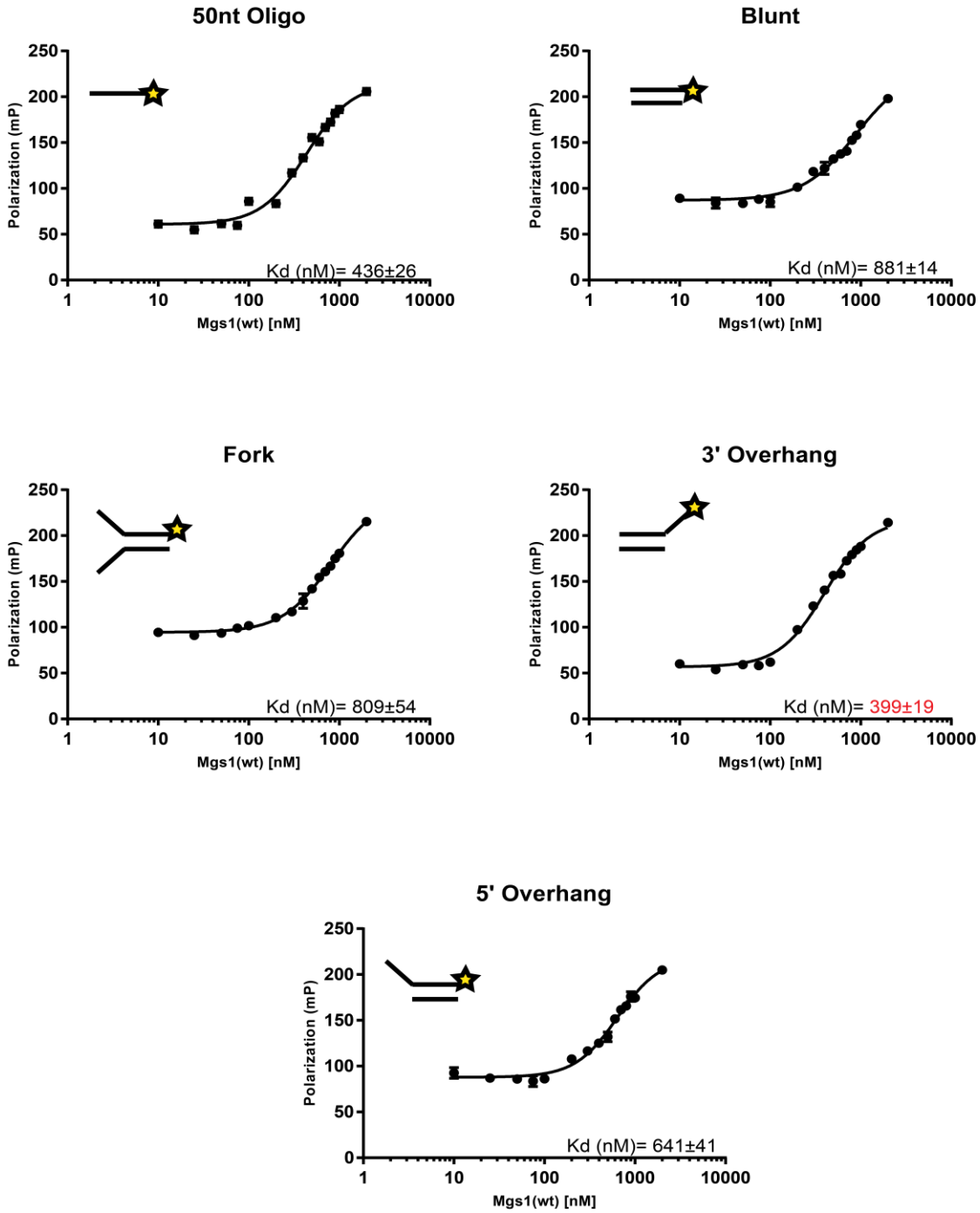


Figure 2.8: **DNA binding of Mgs1 (Simple structures)**: DNA binding of Mgs1 for different structures is shown. The graphical representation of the substrate with the fluorophore is depicted. K_d values in nM are reported in the inset.

Table 2.3: Dissociation constants for DNA binding of Mgs1 (Complex structures)

	DNA structure	K_d (nM)
1	**Lagging strand**	362±30
2	**Leading strand**	519±23
3	**Flapped End**	547±34

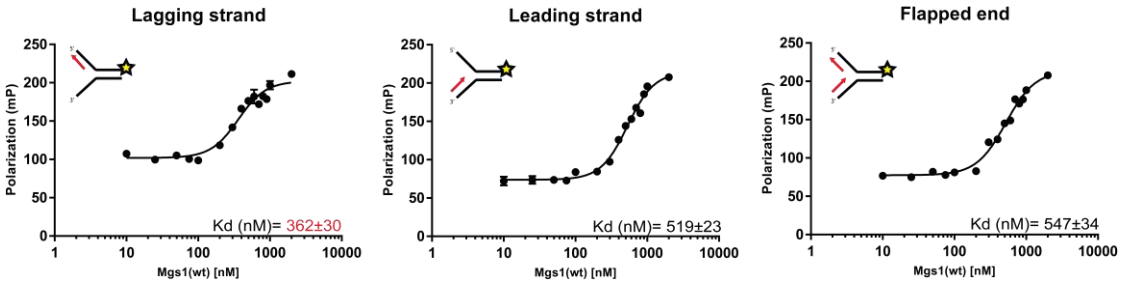


Figure 2.9: **DNA binding of Mgs1 (Complex structures)**: DNA binding of Mgs1 for different structures is shown. The graphical representation of the substrate with the fluorophore is depicted. K_d values in nM are reported in the inset.

Interestingly we found that the complex structure bearing the 3' overhang signature also showed binding preference by Mgs1. The dissociation constant for the lagging strand was found to be 362±30 nM, similar to the *K*d of 399±19 nM for the 3' overhang.

Chapter 3

DISCUSSION

3.1 Mgs1: AAA+ ATPase

Mgs1 is 66.5 kDa protein and is a member of AAA+ ATPase superfamily. The ATPase domain of Mgs1 might be central to Mgs1's physiological role of mediating genome stability in absence of genotoxic agents. The ATPase is a conserved module of approximately 230 amino acids, consisting of the Walker A, Walker B and sensor I and II motif sequences as described earlier in this thesis. Mgs1 is well conserved in prokaryotes and eukaryotes, Werner helicase interacting protein (WRNIP1) being the human homologue. A crystal structure is available for the bacterial homologue; MgsA[25]. The proteins of this family usually form "ring-shaped oligomeric complexes" but are widely diverse in function[14]. MgsA crystal structure reveals a homotetrameric arrangement of the protein, encouraging the notion that Mgs1 might also be an oligomer or form oligomeric structures on DNA.

3.2 Mgs1's ATPase Activity is Influenced to Varying Degree by Different DNA Structures

Mgs1 has a significant stimulation in the ATPase activity in presence of DNA (Figure 2.2 B) as reported earlier[19]. The rate of the ATP hydrolysis increases by about 5 folds upon the addition of DNA in a NADH Enzyme-linked ATPase assay. We

interpret this increase in the activity in the presence of DNA as physical interaction between DNA and Mgs1, nature of which remains unknown.

Studies have shown that ATPase activity of Mgs1 is highly stimulated by a single stranded DNA (ssDNA)[19]. In our ATPase assay we found that the activity was least stimulated by ssDNA (Figure 2.4 B) and most by DNA substrates having both single and double stranded character. These contrasting findings are interesting. The differences in the two results might be due to the fact that in the earlier publication, M13mp18 (ssDNA) or M13mp18 RFI (dsDNA) have been used to test the activity. These M13 ssDNA circles have been known to have regions that conform into a secondary hairpin structure[26]. Our study is more comprehensive with more defined DNA structures. We hypothesize that the ss-ds composite DNA structure provides for a functionally productive confirmation of Mgs1 on DNA, hence stimulating the ATPase activity significantly more than the other structures.

Such ss-ds junctions are abundantly found in stalled replication forks due to polymerase uncoupling and therefore may be the basis of interaction of the first responders of stalled replication forks[27]. Structure based stimulation of ATPase activity has been observed earlier for many DNA Replication repair proteins, SMARCAL1 being an important one[21,28].

3.3 DNA Binding Specificities of Mgs1

DNA Binding affinity is an important factor for the processivity of DNA repair proteins. Structural signature of a stalled replication fork is the first SOS signal for the recruitment of repair proteins. First responders of stalled replication forks recognize

DNA signatures like DSBs which causes recruitment and downstream checkpoint activation and an appropriate DNA damage response pathway[27,29].

RecG is a helicase *Escherichia coli*, involved in DNA repair and recombination. Stalled replication fork can be resolved by formation of a Holliday junction in the recombination related DNA damage pathways. RecG has been shown to preferentially bind and unwind the lagging strand on the replication fork to promote formation of Holliday junctions[24].

Structure specific DNA binding activities have been observed for T4 single stranded binding (SSB) protein, *E.coli* single stranded binding protein, and hRPA (replication protein A). It has been demonstrated earlier that these proteins have different binding preference for the DNA structural substrates. The T4 SSB protein preferentially binds substrates with 5'overhangs, whereas the E. coli SSB protein and human RPA show a preference for substrates with 3' overhangs[30].

It is interesting that such preference of 3' overhang is also shown by Sgs1 a member of the RecQ DNA helicase family[31]. Sgs1 is a homologue of WRN helicase in *Saccharomyces cerevisiae*. Mgs1 and Sgs1 have been implicated in similar pathways and have been thought to interact with Polymerase δ (polymerase of the lagging strand) to counter defects in replication[32]. With these findings in retrospect, it is interesting to investigate if Mgs1 shows any preference on binding for DNA structure substrates.

Our binding studies show that there is a clear difference in binding affinities of Mgs1 for different DNA structural signatures. Mgs1 shows highest binding affinity for DNA structures harboring a 3' overhang, as is apparent from a K_d of 399±19 nM, lower than all the other structures tested in our experimental setup. The important

observation is that Mgs1 binds to each structure, but with different binding affinities. This in is line with the observation from an earlier study[20], which has demonstrated that Mgs1 binds to each tested structure albeit different affinities. Also it is clear from their findings that Mgs1 has significant higher binding for a DNA harboring the 3' overhang regions. These findings encouraged us to further study more complex structures representative of stalled replication fork as used by several earlier studies. We made the similar observation for these complex structures as was the case with simple structures. For a lagging strand substrate the K_d was found to be 362±30 nM, lower than other substrates tested for.

These results suggest that the primary recognition of stalled replication fork by Mgs1 is though recognition and binding to specific structural intermediates presented by stalling of replication. Our hypothesis is that the stalled replication fork is prevented from collapsing by localization of Mgs1 on the fork, but how this action is mediated remains unknown. It is demonstrated earlier that Mgs1 physically interacts with Polymerase δ[33]. The study also suggests that PCNA and Mgs1 share the same interaction sites of polymerase δ. It would be very interesting to study how these three proteins regulate the replication progression in events of no apparent DNA damage.

3.4 Co-operative DNA Binding of Mgs1

The binding curves demonstrates a cooperative binding of Mgs1 on DNA at equilibrium. A cooperative binding behavior is an indication of the oligomeric assembly of proteins[34,35]. Extending the observation further, it might suggest that Mgs1 might form oligomers in presence of DNA. MgsA, the bacterial homolog of Mgs1, is found to be a homotetramer[25]. Future studies may elucidate the exact nature

of the interaction and the changes in the oligomeric status of Mgs1 upon addition of DNA, if any.

3.5 Summary

It is known that Mgs1 and *RAD6* epistasis group proteins are required, specifically the Rad18/Rad5 error free branch, for normal growth[33,36,37]. From the double mutants studies it is clear that *mgs1*Δ causes synthetic lethality with *rad6Δ*. Additionally *mgs1*Δ and *rad5*Δ shows a growth defect.

The cells harboring *mgs1*Δ and *rad5*Δ become sensitive to the treatment of HU but not treatment of UV/MMS. These results suggest that Mgs1 is only involved in resolving stalled replication and not in any other pathway of DNA damage response. This makes Mgs1 the factor important in maintaining genomic stability.

Hydroxyurea (HU) treatment impedes with replication resulting into stalled replication forks. This is due to the effect of HU on the deoxyribonucleotide pools. The deoxyribonucleotide pools are depleted causing immediate stalling of replication forks[38]. These stalled replication forks are known to be stable for a long time before removal of the block, following which the replication can be restarted and resolved by mediation of Rad51[39,40].

We hypothesize that in in events of the stalled replication fork, Mgs1 is recruited on the ssDNA gap on the lagging stand, stabilizing the replication complex. In this light it is possible that Mgs1 is a gatekeeper and maintains a steady rate of replication even under transient impedance of the replication fork. We postulate that Mgs1 has two distinct functions; one, during normal replication fork progression

mediated through direct interaction with the DNA and second, during DNA damage mediated by recruitment on the damage site via UBZ domain.

We delineate a possible mechanism underlying the activities mediated by DNA binding and ATPase activity of Mgs1. Mgs1 identifies the stalled replication fork and gets recruited to the lagging strand fork by structural identification of the DNA or by interaction with RPA and stabilizes the stalled replication fork. It is known that stalled replication forks have extensive ssDNA gaps which might be annealed by Mgs1[13].

Preliminary analysis of the effect of ATP on DNA binding of Mgs1 shows that the ATP binding and hydrolysis might alter the tertiary structure of Mgs1, either making it more active on the fork or dissociate from the fork indicated by changes in the dissociation constant. The precise nature of the effect should be studied in detail. The fork might be resolved and restarted by the Rad6/Rad18/Rad5 pathway downstream of Mgs1. It remains to be evaluated if there is a legitimate physical interaction between Rad5 and Mgs1 and whether they function is the same pathway. Rad6/Rad18 pathway is involved in polyubiquitination of PCNA and from earlier studies we know that Mgs1 does not affect the ubiquitination of PCNA[37] so it is likely that it acts in stabilizing the fork without impeding with the downstream processes.

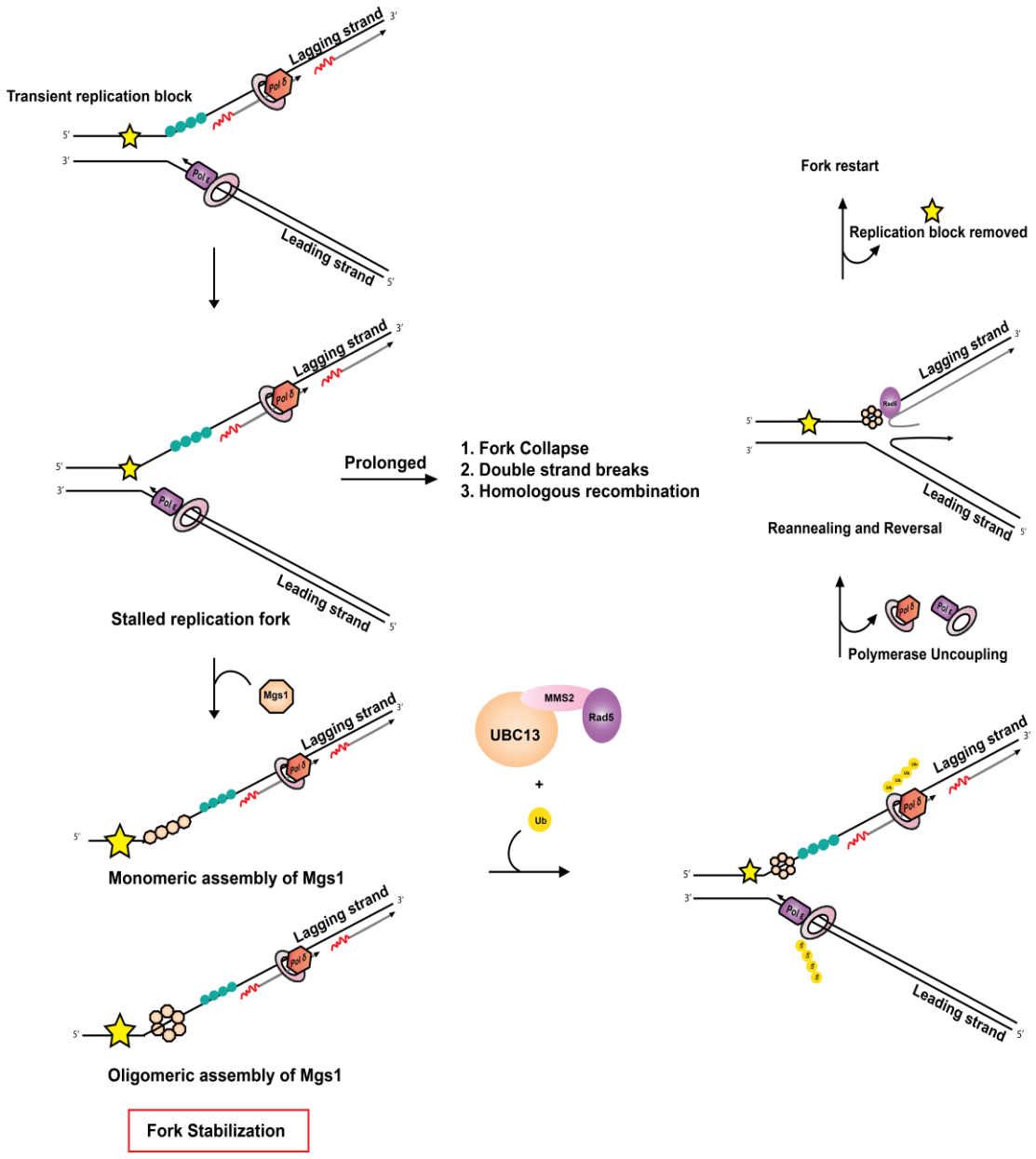


Figure 3.1: **The proposed mechanism of Mgs1 on transiently stalled replication forks**

3.6 Is Mgs1 the Functional Ortholog of SMARCAL1?

Human SMARCAL1 is the member of the SNF2 family ATPases. SMARCAL1 is also a DNA-dependent ATPase. It has been well characterized biochemically thus far, but the yeast ortholog remains unknown[41]. We propose that Mgs1 might be the yeast functional ortholog of SMARCAL1.

SMARCAL1 does not have any helicase activity but is shown to function as an annealing helicase[42], same has been noted about Mgs1 that it has no helicase activity but anneals ss-complementary DNA[19]. The assay used in the paper to determine the annealing activity of Mgs1 uses long heat denatured pUC19 plasmid to assay the annealing by Mgs1, which might be less informative. The annealing activity can be further confirmed by using similar assays for Mgs1, as those used for SMARCAL1, with well-defined DNA substrates.

The ATPase activity of SMARCAL1 is stimulated to various degrees by different DNA sturctures[21,41], similar to what we observe in our ATPase assay for Mgs1. The activity is least stimulated by structures having ss-DNA and most by the DNA containing both ss and ds signature.

SMARCAL1 is shown to be more active toward the lagging strand substrate in the fork regression activity[43]. Our DNA binding assay suggests that Mgs1 has a greater binding for the lagging strand substrate. Functional assay of Mgs1 would be very informative to show whether Mgs1 specific activity is stimulated the same way by lagging strand substrate as for the specific activity of SMARCAl1.

SMARCAl1 is recruited to the fork via RPA. MgsA, the *E.coli* homolog of Mgs1, physically interacts with SSB[25]. Many other studies suggest RPA/SSB

mediated recruitment of DNA damage repair proteins. Recently it has been shown that RecG is recruited by SSB on the fork[44].

Our preliminary data suggests that Mgs1 physically interacts with yRPA in a nickel affinity based assay. These findings might suggest that RPA recruits Mgs1 on the sites of stalled replication fork. Future studies might shed more light in this respect.

ZRANB3 is also a member of the SNF2 family. The recruitment of ZRANB3 on the replication fork is through the interaction via the UBZ domain[45]. There is no known homolog of ZRANB3 in lower eukaryotes[46]. We hypothesize that Mgs1 might be the functional homolog to ZRANB3 and SMARCAL1 and the function has diverged through evolution. It is also very interesting to note that WRNIP (the human homolog of Mgs1) co-localizes with ZRANB3 at the sites of DNA damage[45].

In the light of the evidence presented above it would be interesting to functionally characterize Mgs1 and further analyze the evolutionary pattern of these proteins.

Chapter 4

MATERIALS AND METHODS

4.1 Expression and Purification of Mgs1 (wt) and Mgs1 (K183A)

Expression: Mgs1 (wt) was cloned and expressed by Kun Yang. Mgs1 (K183A), ATPase mutant, was created by site-directed mutagenesis replacing lysine at 183 amino acid position by alanine. The primers used for site-directed mutagenesis are enlisted in the Table 3.1. Cloned in pET28a plasmid and expressed in the Rosetta DE3 *E.Coli* cell line.

Table 4.1: Primers used for site-directed mutagenesis of Mgs1 (K183A)

Mgs1 (K183A)_Forward	CCT CCA GGT GTA GGA GCG ACT TCA CTA GCT AGA CTA TTA ACG
Mgs1 (K183A)_Reverse	CGT TAA TAG TCT AGC TAG TGA AGT CGC TCC TAC ACC TGG AGG

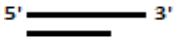
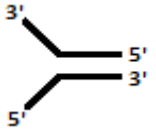
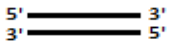
Purification: Purification of Mgs1 (wt) and Mgs1 (K183A) was carried out as described previously[19] with the following changes. Rosetta carrying either the plasmid was grown at 37℃ to OD_{600} of 0.6–0.8 in 2.5-liter LB broth containing

Chloramphenicol (35 μg/ml) and Kanamycin (50 μg/ml). Isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM at 16℃ overnight. Cells were harvested by centrifugation. The cells were re-suspended in lysis buffer (buffer A) [50 mM Sodium Phosphate (pH 7.0)]/10 mM 2-mercaptoethanol/10% glycerol/50 mM NaCl]. Cells were sonicated and centrifuged at 18000×g for 30mins to collect the cell extract. Supernatant was collected and Polymin P was added to a final concentration of 0.05% (wt/vol). The suspension was stirred for 30mins at 4℃. The supernatant was separated by centrifugation at 18000×g for 10mins. To the resultant supernatant Ammonium sulphate was added to a final concentration of 50% to precipitate proteins. Stirred for 30mins and centrifuged to collect the precipitated proteins. Precipitate was resuspended in buffer B [50mM sodium phosphate (pH 7.0)/300mM NaCl/10mM 2-mercaptoethanol/100mM Imidazole]. TALON metal affinity resin (Taraka Clontech) was washed and equilibrated with the buffer B. The proteins were allowed to bind to the resin for 2 hours at 4℃. The resin was applied to a column and washed with the buffer B followed by wash with Buffer C [50mM sodium phosphate (pH 7.0)/800mM NaCl/10mM 2-mercaptoethanol/100mM Imidazole]. Mgs1 was eluted with the elution buffer (buffer D) [50 mM Sodium Phosphate (pH 7.0)]/10 mM 2-Mercaptoethanol/10% glycerol/50 mM NaCl/500mM Imidazole]. The eluted fractions were concentrated and dialyzed in the storage buffer [50mM Sodium Phosphate (pH 7.0)/10% glycerol/50mM NaCl/2mM DTT] overnight and stored at -80℃. Mgs1 concentration was determined by the Bradford method (Bio-Rad).

4.2 NADH Enzyme-Linked ATPase Assay

The ATPase activity of Mgs1 (wt) and Mgs1 (K183A) was determined using a phosphoenolpyruvate kinase/lactate dehydrogenase-coupled assay [47,48] at 25°C in a buffer containing [25 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$/25 mM NaCl/ 1 mM ATP/4 mM Phosphoenolpyruvate (PEP)/400 µM NADH and 3 units of Phosphoenolpyruvate kinase-Lactate dehydrogenase mix (Sigma)]. Assay volume was 60ul in a quartz cuvette. Mgs1 (wt or K183A) as indicated were incubated with DNA-template structures and the rates of ATP hydrolysis were determined by measuring the UV absorbance change at 340 nm.

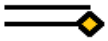
Table 4.2: Oligonucleotides used to generate DNA structure templates: ATPase Assay

	Name	Sequence (5'-3')	Structure
1	50nt Oligo	GCA TCG TAT AGC GCC GTT TTT TTT TTT TTT TTT T	
2	19nt Oligo	GCA TCG TAT AGC GCC GGC G	
3	5' Overhang	1. GCA TCG TAT AGC GCC GTT TTT TTT TTT TTT TTT TTT T 2.CGC CGG CGC TAT ACG ATG	
4	3' Overhang	1. TTT TTT TTT TTT TTT TTT TTT GCA TCG TAT AGC GCC GGC G 2. CGC CGG CGC TAT ACG ATG	
5	Fork	1. CGC CGG CGC TAT ACG ATG CTT TTT TTT TTT TTT TTT TTT T 2. TTT TTT TTT TTT TTT TTT TTT GCA TCG TAT AGC GCC GGC G	
6	Blunt	1. CAG TCT TCA CCT ATA AAC TCT GAG AAG AGA GAA AAC TTG 2. CAA GTT TTC TCT CTT CTC AGA GTT TAT AGG TGA AGA CTG	

4.3 Preparation of DNA Substrates for Fluorescence Polarization Assay

DNA labelled at the 3'end with 6-FAM (Fluorescein) (Integrated DNA Technology, IDT) was acquired. DNA structures were created by annealing the 6-FAM labelled-oligonucleotide with different DNA oligonucleotide. The DNA oligonucleotides, as indicated (1.1 Table), were incubated in 1:1 ratio in the annealing buffer [10 mM Tris (pH 7.5)/ 50 mM NaCl/ 1 mM EDTA]. Incubated for 3mins at 95°C and allowed to cool at R/T for 2 hours and stored in -20°C till further use. The DNA-template structures used for this study are listed (3.3 Table).

Table 4.3: Oligonucleotides used to generate DNA structure templates: FP Assay

	Name	Sequence (5'-3')	Structure (Oligo's used)
1	FAM-Oligo	TGC CAG TTC ACA TCA GAA TCG CCT AGC TCG ACG CCA TTA ATA ATG TTT TC-FAM	 *6-FAM
2	3' Overhang	CGA GCT AGG CGA TTC TGA TGT GAA CTG GCA	 (1.2)
3	5' Overhang	GAA AAC ATT ATT AAT GGC GTC GAG CTA GGC	 (1,3)
4	Blunt end	GAA AAC ATT ATT AAT GGC GTC GAG CTA GGC GAT TCT GAT GTG AAC TGG CA	 (1,4)
5	Fork	GAA AAC ATT ATT AAT GGC GTC GAG CTA GGA AAA CAT TAT TAA TGG CGT CGA GCT AG	 (1,5)
6	Leading Strand	CTA GCT CGA C	 (1,5,6)
7	Lagging Strand	TGA ACT GGC A	 (1,5,7)
8	Flapped Structure	6. CTA GCT CGA C 7. TGA ACT GGC A	 (1,5,6,7)

4.4 Fluorescence Polarization Assay

4.4.1 FP Assay for Mgs1 Binding

Fluorescence Polarization assay was performed to understand binding interaction between DNA-Mgs1 under different conditions. The assay was performed in the FP assay buffer [25mM Tris (pH 7.5)/ 5mM $MgCl_2$/ 1mM DTT]. Mgs1 was incubated with DNA structure substrate, in concentrations as indicated, at room temperature in a total reaction volume of 60 µl. The reactions were performed in 96-well opaque plate (Corning) and equilibrated for 30 min before readout. The change in Fluorescence polarization (ΔmP) was measured at 25°C using a plate reader (PerkinElmer) with 485 nm excitation and 535 nm emission wavelengths. The dissociation constants (K_d) were determined by a one site-specific binding with Hill slope analysis using the program Prism 6.05 (GraphPad Inc), as illustrated below. Data is averaged over three repeats for each condition.

$$Y = \frac{Bmax \times X^h}{Kd + X^h} + Background$$

Where,

Y= Fluorescence Polarization (mP)

X= Concentration of Mgs1 (nM)

Bmax= Maximum specific binding

h= Hill Slope

4.4.2 FP Assay for RPA Binding

RPA (Replication protein A) was used as a standard control for the Fluorescence polarization assay. Purified RPA was incubated with the 100nM 6-FAM

labelled 50nt oligonucleotide in increasing concentration from 0nM to 500nM in the buffer described in section 4.4.1. The reactions were performed in 96-well opaque plate (Corning) and equilibrated for 30 min before readout. The fluorescence polarization (ΔmP) was measured at 25°C using a plate reader (PerkinElmer) with 485 nm excitation and 535 nm emission wavelengths. The dissociation constants (K_d) were determined by a one site-total binding analysis using the program Prism 6.05 (GraphPad Inc).

4.4.3 Competitive Binding Assay

A qualitative competition based FP assay was performed as a control for the DNA binding of Mgs1. 1.4 uM of Mgs1 was incubated with 100 nM 6-FAM labelled 50nt oligonucleotide to achieve saturation binding in the buffer described in section 4.4.1. Increasing concentration (0nM-6uM) of unlabeled 50nt Oligonucleotide, with the same sequence as the labelled counterpart, was added in each binding reaction and incubated for 30 min before readout. The data was fit to a sigmoidal equation from Prism 6.05 (GraphPad Inc).

REFERENCES

1. Wilson, John H.,,Hunt, Tim,,. *Molecular biology of the cell, 4th edition : A problems approach.* New York; London: Garland Science; 2002.

2. Huang D, Piening BD,Paulovich AG,. The preference for error-free or error-prone postreplication repair in saccharomyces cerevisiae exposed to low-dose methyl methanesulfonate is cell cycle dependent. *Mol Cell Biol*. 2013;33(8):1515-27.

3. Recolin B, van der Laan S, Tsanov N,Maiorano D,. Molecular mechanisms of DNA replication checkpoint activation. *Genes*. 2014;5(1):147-75.

4. Yeeles JT, Deegan TD, Janska A, Early A,Diffley JF,. Regulated eukaryotic DNA replication origin firing with purified proteins. *Nature*. 2015;519(7544):431-5.

5. Lee J, Chastain PD 2nd, Kusakabe T, Griffith JD,Richardson CC,. Coordinated leading and lagging strand DNA synthesis on a minicircular template. *Mol Cell*. 1998;1(7):1001-10.

6. Burgers PM,. Polymerase dynamics at the eukaryotic DNA replication fork. *The Journal of biological chemistry*. 2009;284(7):4041-5.

7. Morrison A, Bell JB, Kunkel TA,Sugino A,. Eukaryotic DNA polymerase amino acid sequence required for 3'----5' exonuclease activity. *Proc Natl Acad Sci U S A*. 1991;88(21):9473-7.

8. Morrison A, Johnson AL, Johnston LH,Sugino A,. Pathway correcting DNA replication errors in saccharomyces cerevisiae. *EMBO J*. 1993;12(4):1467-73.

9. Fijalkowska IJ, Schaaper RM,Jonczyk P,. DNA replication fidelity in escherichia coli: A multi-DNA polymerase affair. *FEMS Microbiol Rev*. 2012;36(6):1105-21.

10. Ciccia A ES. The DNA damage response: Making it safe to play with knives. *Mol Cell*. 2010;40(2):179-204.

11. Curtin NJ,. DNA repair dysregulation from cancer driver to therapeutic target. *Nature reviews.Cancer*. 2012;12(12):801-17.

12. Branzei, D,Foiani, M,. Interplay of replication checkpoints and repair proteins at stalled replication forks. *DNA Repair DNA Repair*. 2007;6(7):994-1003.

13. Petermann E HT. Pathways of mammalian replication fork restart. *Nature reviews.Molecular cell biology*. 2010;11(10):683-7.

14. Ogura T WA. AAA+ superfamily ATPases: Common structure--diverse function. *Genes to cells : devoted to molecular & cellular mechanisms*. 2001;6(7):575-97.

15. Snider, Jamie, Thibault, Guillaume, Houry,Walid A,. The AAA+ superfamily of functionally diverse proteins. *Genome Biol Genome Biology*. 2008;9(4):1-8.

16. Walker JE, Saraste M, Runswick MJ,Gay NJ,. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J*. 1982;1(8):945-51.

17. Hanson PI WS. AAA+ proteins: Have engine, will work. *Nature reviews.Molecular cell biology*. 2005;6(7):519-29.

18. Saugar I, Parker JL, Zhao S,Ulrich HD,. The genome maintenance factor Mgs1 is targeted to sites of replication stress by ubiquitylated PCNA. *Nucleic Acids Res*. 2012;40(1):245-57.

19. Hishida T, Iwasaki H, Ohno T, Morishita T,Shinagawa H,. A yeast gene, MGS1, encoding a DNA-dependent AAA(+) ATPase is required to maintain genome stability. *Proc Natl Acad Sci U S A*. 2001;98(15):8283-9.

20. Kim JH, Kang YH, Kang HJ, Kim DH, Ryu GH, Kang MJ,Seo YS,. In vivo and in vitro studies of Mgs1 suggest a link between genome instability and okazaki fragment processing. *Nucleic Acids Res*. 2005;33(19):6137-50.

21. Bétous R, Mason AC, Rambo RP, Bansbach CE, Badu-Nkansah A, Sirbu BM, Eichman BF,Cortez D,. SMARCAL1 catalyzes fork regression and holliday junction migration to maintain genome stability during DNA replication. *Genes Dev*. 2012;26(2):151-62.

22. Fan J PN. Structure and conformational change of a replication protein A heterotrimer bound to ssDNA. *Genes Dev*. 2012;26(20):2337-47.

23. Kim C, Snyder RO,Wold MS,. Binding properties of replication protein A from human and yeast cells. *Mol Cell Biol*. 1992;12(7):3050-9.

24. McGlynn P LR. Rescue of stalled replication forks by RecG: Simultaneous translocation on the leading and lagging strand templates supports an active DNA unwinding model of fork reversal and holliday junction formation. *Proc Natl Acad Sci U S A*. 2001;98(15):8227-34.

25. Page AN, George NP, Marceau AH, Cox MM,Keck JL,. Structure and biochemical activities of escherichia coli MgsA. *The Journal of biological chemistry*. 2011;286(14):12075-85.

26. Brosh RM Jr, Karow JK, White EJ, Shaw ND, Hickson ID,Bohr VA,. Potent inhibition of werner and bloom helicases by DNA minor groove binding drugs. *Nucleic Acids Res*. 2000;28(12):2420-30.

27. Cimprich KA CD. ATR: An essential regulator of genome integrity. *Nature reviews.Molecular cell biology*. 2008;9(8):616-27.

28. Muthuswami R, Truman PA, Mesner LD,Hockensmith JW,. A eukaryotic SWI2/SNF2 domain, an exquisite detector of double-stranded to single-stranded DNA transition elements. *The Journal of biological chemistry*. 2000;275(11):7648-55.

29. Sogo JM, Lopes M,Foiani M,. Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science (New York, N.Y.)*. 2002;297(5581):599-602.

30. Delagoutte E, Heneman-Masurel A,Baldacci G,. Single-stranded DNA binding proteins unwind the newly synthesized double-stranded DNA of model miniforks. *Biochemistry*. 2011;50(6):932-44.

31. Bennett RJ, Keck JL,Wang JC,. Binding specificity determines polarity of DNA unwinding by the Sgs1 protein of S. cerevisiae. *J Mol Biol*. 1999;289(2):235-48.

32. Branzei, Dana, Seki, Masayuki, Onoda, Fumitoshi, Yagi, Hideki, Kawabe, Yoh-ichi,Enomoto, Takemi,. Characterization of the slow-growth phenotype of S. cerevisiae whip/mgs1 sgs1 double deletion mutants. *DNA Repair DNA Repair*. 2002;1(8):671-682.

33. Vijehmotlagh, N, Seki, M, Branzei, D,Enomoto, T,. Mgs1 and Rad18/Rad5/Mms2 are required for survival of saccharomyces cerevisiae mutants with novel temperature/cold sensitive alleles of the DNA polymerase δ subunit, Pol31. *DNA Repair DNA Repair*. 2006;5(12):1459-1474.

34. Domsic JF, Chen HS, Lu F, Marmorstein R,Lieberman PM,. Molecular basis for oligomeric-DNA binding and episome maintenance by KSHV LANA. *PLoS pathogens*. 2013;9(10).

35. Pray TR, Burz DS,Ackers GK,. Cooperative non-specific DNA binding by octamerizing lambda cI repressors: A site-specific thermodynamic analysis. *J Mol Biol*. 1998;282(5):947-58.

36. Hishida T, Ohno T, Iwasaki H,Shinagawa H,. Saccharomyces cerevisiae MGS1 is essential in strains deficient in the RAD6-dependent DNA damage tolerance pathway. *EMBO J*. 2002;21(8):2019-29.

37. Hishida T, Ohya T, Kubota Y, Kamada Y,Shinagawa H,. Functional and physical interaction of yeast Mgs1 with PCNA: Impact on RAD6-dependent DNA damage tolerance. *Mol Cell Biol*. 2006;26(14):5509-17.

38. Bianchi V, Pontis E,Reichard P,. Changes of deoxyribonucleoside triphosphate pools induced by hydroxyurea and their relation to DNA synthesis. *The Journal of biological chemistry*. 1986;261(34):16037-42.

39. Sirbu BM, Couch FB, Feigerle JT, Bhaskara S, Hiebert SW,Cortez D,. Analysis of protein dynamics at active, stalled, and collapsed replication forks. *Genes Dev*. 2011;25(12):1320-7.

40. Petermann, E., Orta, M.L., Issaeva, N., Schultz, N.,Helleday, T.,. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol Cell*. 2010;37(4):492-502.

41. Bansbach CE, Bétous R, Lovejoy CA, Glick GG,Cortez D,. The annealing helicase SMARCAL1 maintains genome integrity at stalled replication forks. *Genes Dev*. 2009;23(20):2405-14.

42. Yusufzai T KJ. HARP is an ATP-driven annealing helicase. *Science (New York, N.Y.)*. 2008;322(5902):748-50.

43. Betous, Remy, Couch, Frank B., Mason, Aaron C., Eichman, Brandt F., Manosas, Maria,Cortez, David,. Substrate-selective repair and restart of replication forks by DNA translocases. *Cell Reports Cell Reports*. 2013;3(6):1958-1969.

44. Sun Z, Tan HY, Bianco PR,Lyubchenko YL,. Remodeling of RecG helicase at the DNA replication fork by SSB protein. *Scientific reports*. 2015;5.

45. Ciccia A, Nimonkar AV, Hu Y, Hajdu I, Achar YJ, Izhar L, Petit SA, Adamson B, Yoon JC, Kowalczykowski SC, Livingston DM, Haracska L,Elledge SJ,. Polyubiquitinated PCNA recruits the ZRANB3 translocase to maintain genomic integrity after replication stress. *Mol Cell*. 2012;47(3):396-409.

46. Ulrich HD TT. Readers of PCNA modifications. *Chromosoma*. 2013;122(4):259-74.

47. Yang, Kun, Gong, Ping, Gokhale, Parikshit,Zhuang, Zhihao,. Chemical protein polyubiquitination reveals the role of a noncanonical polyubiquitin chain in DNA damage tolerance. *ACS Chem.Biol.ACS Chemical Biology*. 2014;9(8):1685-1691.

48. The structure of a ring-opened proliferating cell nuclear antigen–replication factor C complex revealed by fluorescence energy transfer.