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The Role of DMC1 in Meiotic Homologous Recombination in *Arabidopsis Thaliana*

Daniel Hiott

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Daniel Hiott*, Garrett Buzzard, and Dr. Michael Sehorn

Abstract

Dmc1 is a meiosis-specific recombinase that is integral to DNA double-strand break (DSB) repair using the homologous recombination pathway. This pathway uses a homologous sister chromatid as a template for DNA repair synthesis. After a DSB is formed, DNA end resection occurs, leaving a 3'single strand DNA (ssDNA) overhang. Dmc1 polymerizes on this ssDNA to form a right-handed helical protein filament that catalyzes the search for a homologous DNA region to facilitate strand invasion. Other protein factors such as Hop2-Mnd1 are recruited to promote Dmc1 mediated recombination. Recently, a mutation in Dmc1 was found to cause sterility in *Arabidopsis thaliana*. To determine the impact this mutation has on the recombination activities of Dmc1, I expressed and purified wildtype and mutant *at*Dmc1 proteins from bacteria. The purified wildtype and variant Dmc1 proteins were subjected to various biochemical analyses that monitored the ability of Dmc1 to catalyze the search for homology and perform DNA strand exchange. The variant Dmc1 protein was severely attenuated in its ability to perform homology directed repair. Protein factors such as Hop2-Mnd1 and CAF1G in *Arabidopsis thaliana* potentially modulate the activity of Dmc1 and may be able to compensate for this loss of activity. The results from this project provide insight into the function of a highly conserved amino acid residue within Dmc1 and may explain why a mutation of this amino acid results in sterility in plants.

Introduction

Meiosis is an essential process in most eukaryotic organisms to produce haploid gametes for reproduction. The Spo11 enzyme initiates meiosis by generating controlled DNA double-strand breaks (DSBs) across the genome (1). These DSBs are then repaired through the homologous recombination DNA repair pathway (2), forming a physical connection between the homologous chromosomes. Following the DSB, the 3' ends of the dsDNA are processed to produce 3' ssDNA overhangs, which are the site for recombinases such as Dmc1 to bind (3). Dmc1 forms a right-handed filament on this overhand to form a nucleoprotein filament (4), and this filament searches for homology by invading the homologous chromosome. When this homology is found, Dmc1 catalyzes DNA pairing between the homologous and complementary strand, and the complementary strand is displaced to form a displacement loop, or D-loop. This formation is followed by strand exchange. The repair synthesis restores the lost genetic information from the DSB, and Holiday junctions are formed between the D-loop. These junctions are then resolved to create crossover or non-crossover products.

Dmc1 plays an important role in meiosis, and DMC1 -/- knockout mice live but are sterile (5). The activity of Dmc1 is aided by accessory factors such as Calcium ions (5) and Hop2-Mnd1 (6). Hop2-Mnd1 (H2M1) is a heterodimer which interacts with Dmc1 to aid in the formation of D-loops by stabilizing the Dmc1 nucleoprotein filament that recruits the dsDNA templates which are searched for homology (7). DMC1 is very well conserved across eukaryotic species, and many of the amino acids are identical, especially in a domain known as the YARA

box (8). Mutations in this region often lead to sterility. A sterile *Arabidopsis thaliana* from Dr. Gregory Copenhaver's lab at the University of North Carolina Chapel Hill was screened for mutations. It was determined that there was an A200V mutation in the *at*DMC1 gene, and this plasmid was sent to Dr. Michael Sehorn's lab at Clemson University which specializes in homologous recombination and double strand break repair. This mutation changes an alanine to a valine at the last A of the YARA box. This amino acid change leads to an increased area of hydrophobicity and an increased size at this location. This change may lead to an inability of the *at*DMC1 protein to catalyze D-Loop formation, due to an inability to bind DNA, inability to form a filament on DNA, or an inability to hydrolyze ATP. These functions are the topic of study in this paper.

Arabidopsis thaliana is one of the best model organisms for flowering plants in identifying genes and determining their function (9). It has a rather small 125 megabase genome, and it was the first complete plant genome sequence (9). In addition, its short generation time, many offspring, and small size make it preferable for studies in flowering plants which can then be extrapolated to other organisms (10). *Arabidopsis* thus serves as a beneficial model organism to study the impact of a mutation in the YARA domain of DMC1 protein, with implications for basic research and potential fertility treatments.

Materials and Methods

Plasmids

The Arabidopsis expression plasmids were a gift from Gregory Copenhaver's lab at the University of North Carolina Chapel Hill.

Purification of atDmc1$_{WT}$ and atDmc1$_{A200V}$.

The $(HIS)_6$-*at*Dmc1$_{WT}$ pET11d expression plasmid was transformed into competent *E. coli* strain Rosetta (DE3) cells. A 300 mL culture was created from the transformed cells using 1X LB, 1μM Ampicillin, and 1μM Chloramphenicol. This was shaken overnight at 37ºC. 50 mL of this culture was added to each of six flasks containing 1.6 L 2XLB, 1μM Ampicillin, and 1μM Chloramphenicol. This grew at 37ºC until the OD measured 0.8. Expression of *at*Dmc1$_{WT}$ was induced with 1mM IPTG at 16ºC for 20 hours. The cells were harvested by centrifugation at 5000 rpm. Cell pellets were lysed with Buffer A (50 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA),10% sucrose, 1 mM beta-mercaptoethanol (BME), 0.01% Igepal, 1 mM benzamide, 10 μg/ml lysozyme, 1 mM phenylmethylsulphonyl fluoride, and protease inhibitors: aproptinin, leupeptin, chymostatin and pepstatin A (5ug/ml)) containing 250 mM KCl and 300 mM Na_2SO_4. Cells were resuspended and sonicated for six 30 second cycles at 80% amplitude. This lysate was centrifuged at 40,000 rpm for 90 minutes in Beckman Type Ti45 rotor at 4ºC. The supernatant of the lysate was incubated with 1 mL Ni-NTA agarose beads overnight. The mixture was then washed with 25 mL Buffer B (20mM KH_2PO_4 pH 7.5, 10% glycerol, 0.5mM EDTA), 1 M KCl, and 25 mM imidazole. A second wash was performed with 25 mL of Buffer B with 150 mM KCl and 25 mM imidazole. The protein was eluted with Buffer B containing 150 mM KCl and 500 mM imidazole. The fractions containing $(HIS)_6$-*at*Dmc1$_{WT}$ were loaded onto a 2 mL Macro hydroxyapatite column. The protein was eluted with a 12 mL gradient of Buffer B with 1 mM dithiothreitol (DTT) and 0.01% Igepal from 0-120 mM

KH_2PO_4 then 20 mL of Buffer B at 120 mM KH_2PO_4 followed by a 28 mL gradient of Buffer B from 120-400 mM KH_2PO_4. The peak fractions at 120 mM KH_2PO_4 were pooled, concentrated to 250 µL using a 30K MWCO Amicon filter tube, and then loaded onto a 30 mL S-200 column. The protein was eluted with Buffer B containing 300 mM KCl, then peak fractions were pooled, spun in a 30K MWCO Amicon filter tube to reach a concentration of 7 mg/mL. 2.5 µL aliquots of purified $(HIS)_6$-*at*Dmc1$_{WT}$ were frozen for storage at -80ºC.

atDmc1$_{A200V}$ was also purified as described above.

Purification of Hop2-Mnd1

Murine Hop2-Mnd1 was purified using methods previously described in (9).

Electrophoretic Mobility Shift Assay (EMSA).

To test for ssDNA binding ability, *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ at increasing concentrations (0.25 µM, 0.5 µM, 0d.75 µM, 1.0 µM, 1.25 µM, 1.5 µM, 1.75µM, and 2.0 µM) was incubated with ^{32}P-labeled oligonucleotide OL90 (1.2 µM nucleotides) in Buffer C (25 mM Tris-HCl pH 7.4, 0.1 µg/µl BSA, 1 mM DTT, 2 mM ATP, 1.2 mM $MgCl_2$) for 10 min at 37ºC. DNA loading dye (10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 50% glycerol, 0.1% (w/v) Orange G) was added to each reaction and the samples were resolved with 10% non-denaturing polyacrylamide gel electrophoresis. Gels were dried on cellulose chromatography paper and analyzed with a phosphorimager.

To test for dsDNA binding ability, *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ at increasing concentrations (0.25 μM, 0.5 μM, 0d.75 μM, 1.0 μM, 1.25 μM, 1.5 μM, 1.75μM, and 2.0 μM) was incubated with radio-labeled duplex DNA (3.2 μM nucleotides) in Buffer C (25 mM Tris-HCl pH 7.4, 0.1 μg/μl BSA, 1 mM DTT, 2 mM ATP, 1.2 mM $MgCl_2$) for 10 min at 37°C. DNA loading dye (10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 50% glycerol, 0.1% (w/v) Orange G) was added to each reaction and the samples were resolved with 10% non-denaturing polyacrylamide gel electrophoresis. Gels were dried on cellulose chromatography paper and analyzed with a phosphorimager.

D-Loop Activity Assay

To determine the ability of hDMC1$_{WT}$ to perform D-Loop activity, ^{32}P-labeled oligonucleotide OL90 (1.2 μM nucleotides) was incubated with hDMC1$_{WT}$ (1.0 μM) in Buffer C (25 mM Tris-HCl pH 7.4, 0.1 μg/μl BSA, 1 mM dithiothreitol, 2 mM ATP, 1.2 mM $MgCl_2$) for 10 minutes at 37°C. Supercoiled pBluscript (35 μM base pairs) was added, and incubated for 0.5, 3 minutes at 37°C. Reaction was stopped with incubation of SDS (0.8%) and Proteinase K (5 μg/ml) for 30 minutes. DNA loading dye was added and the samples were resolved with 0.9% agarose gel electrophoresis at 80V for 90 minutes. Gels were dried on anion exchange paper and visualized using a phosphorimager and Image Lab Software (BioRad).

To determine the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to perform D-Loop activity, a similar process was followed, with incubation of ^{32}P-labeled oligonucleotide OL90 (1.2 μM

nucleotides) with *at*Dmc1$_{WT}$ (1.0 μM) in Buffer C for 10 minutes at 37ºC. Supercoiled pBluscript (35 μM base pairs) was added, and incubated for 0.5, 3, 8 minutes at 37ºC. Reaction was stopped with incubation of SDS (0.8%) and Proteinase K (5 μg/ml) for 30 minutes. This process was repeated for *at*Dmc1$_{A200V.}$ DNA loading dye was added and the reaction samples were resolved with 0.9% agarose gel electrophoresis at 80V for 90 minutes. Gels were analyzed as described above.

To determine the ability of $CaCl_2$ to stimulate D-Loop activity in *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V,}$ a similar time-course assay was followed. ^{32}P-labeled oligonucleotide OL90 (1.2 μM nucleotides) with *at*Dmc1$_{WT}$ (1.0 μM) in Buffer C for 10 minutes at 37ºC. $CaCl_2$ (2 mM) was added to the reaction and incubated for 5 minutes. Supercoiled pBluscript (35 μM base pairs) was added, and incubated for 0.5, 3, 8 minutes at 37ºC. Reaction was stopped with incubation of SDS (0.8%) and Proteinase K (5 μg/ml) for 30 minutes. This process was repeated for *at*Dmc1$_{A200V.}$ DNA loading dye was added and the reaction samples were resolved with 0.9% agarose gel electrophoresis at 80V for 90 minutes. Gels were analyzed as described above.

To determine the impact of mH2M1 on D-Loop activity in *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V,}$ an endpoint assay was performed. ^{32}P-labeled oligonucleotide OL90 (1.2 μM nucleotides) with *at*Dmc1$_{WT}$ in increasing concentrations (0.38, 0.64, 1.02, 1.36, 1.70, 2.04 μg) in Buffer C for 10 minutes at 37ºC. $CaCl_2$ (2 mM) was added to the reaction and incubated for 5 minutes for the indicated lanes. mH2M1 (0.5 μM) was added and incubated at 37º C for 5 minutes for the indicated lanes. Supercoiled pBluscript (35 μM base pairs) was added and incubated for 15 minutes at 37ºC. Reaction was stopped with incubation of SDS (0.8%) and Proteinase K (5

μg/ml) for 30 minutes. This process was repeated for *at*Dmc1$_{A200V.}$ DNA loading dye was added and the reaction samples were resolved with 0.9% agarose gel electrophoresis at 80V for 90 minutes. Gels were analyzed as described above.

To determine the combined impact of $CaCl_2$ and mH2M1 on D-Loop activity in *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V,}$ an endpoint assay was performed. ^{32}P-labeled oligonucleotide OL90 (1.2 μM nucleotides) with *at*Dmc1$_{WT}$ (1.0 μM) in Buffer C for 10 minutes at 37ºC. $CaCl_2$ (2 mM) was added to the reaction and incubated for 5 minutes. mH2M1 (0.5 μM) was added and incubated for 5 minutes at 37º C for the indicated lanes. Supercoiled pBluscript (35 μM base pairs) was added, and incubated for 0.5, 3, 8 minutes at 37ºC. Reaction was stopped with incubation of SDS (0.8%) and Proteinase K (5 μg/ml) for 30 minutes. This process was repeated for *at*Dmc1$_{A200V.}$ DNA loading dye was added, and the reaction samples were resolved with 0.9% agarose gel electrophoresis at 80V for 90 minutes. Gels were analyzed as described above.

Single Strand Exchange Assay

To test the ability of *at*DMC1$_{WT}$ and *at*DMC1$_{A200V}$ to conduct DNA strand exchange activity, a time course assay was performed. Unlabeled OL90mer (0.5 pmol/μL) was incubated with *at*DMC1$_{WT}$ (1.5 μM) in a Buffer C solution for 10 minutes at 37ºC. $CaCl_2$ (2 mM) was added to the reaction and incubated for 5 minutes. Complementary duplex DNA (3.2 μM base pairs) and spermidine (4 μM) were added to start the reaction. This product was incubated for 15 minutes, 30 minutes, 45 minutes, and 60 minutes respectively. After this addition, the reaction

products were deproteinized by addition of SDS (0.8%) and Proteinase K (5 μg/ml) which were then incubated at 37ºC for 30 minutes. A similar process was conducted for *at*DMC1$_{A200V}$ (1.5 μM). DNA loading dye was added and the samples were resolved with 13% non-denaturing polyacrylamide gel at 110V for 90 minutes. The gel was visualized on a phosphorimager using Image Lab Software (BioRad).

Results

*at*Dmc1$_{WT}$ and *at*DMC1$_{A200V}$ can be Successful Grown and Purified

As described in Methods, protein purification of *at*Dmc1$_{WT}$ and *at*DMC1$_{A200V}$ was conducted so that biochemical analysis could be performed. Expression plasmids gifted to the Sehorn lab from Dr. Gregory Copenhaver were transformed into bacterial cells, grown and purified to isolate the proteins. After being run through columns (Ni-NTA and Macrohydroxyapatite), *at*Dmc1$_{WT}$ and *at*DMC1$_{A200V}$ were identified as 37.5 kDa bands on a 13% SDS-polyacrylamide gel after electrophoresis and staining with Coomassie Blue (**Figure 1**). This indicates that the proteins were successfully grown and purified.

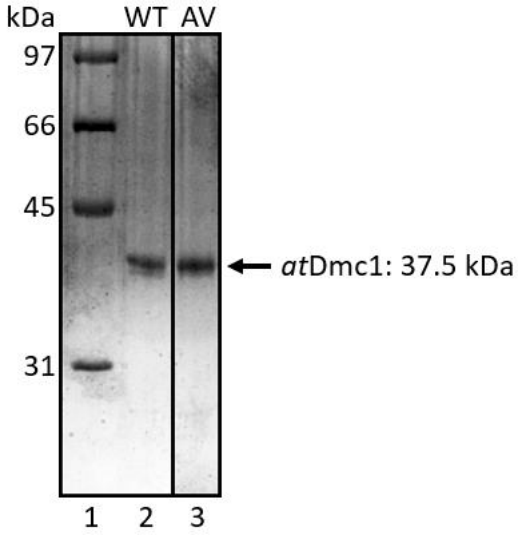

Figure 1. Coomassie Stain Concentration Check of Purified *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$

***at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ can bind to ssDNA**

To test the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to bind single-stranded DNA, an EMSA was performed by incubating single-stranded ^{32}P-labeled ssDNA with purified *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ at increasing concentrations **(Figure 2)**. Without the presence of protein (lane 1), no protein-ssDNA complex was formed. The unbound ssDNA appeared near the bottom of the gel. As the concentration of *at*Dmc1$_{WT}$ was increased, a protein-ssDNA complex was formed (lanes 2-5). The larger size of this complex causes it to not migrate as quickly on a polyacrylamide gel. Similarly, in the absence of protein, the unbound ssDNA did not form a protein-ssDNA complex (lane 6). As the concentration of *at*Dmc1$_{A200V}$ increased, a protein-ssDNA complex was also formed (lanes 7-10). Both *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ can bind to ssDNA, but further work will need to be done to optimize the EMSA conditions.

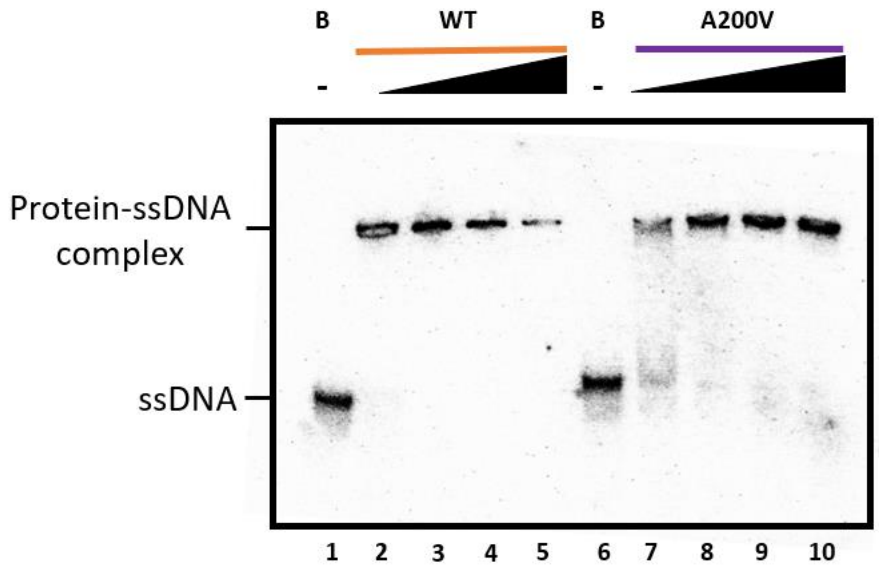

Figure 2. Electrophoretic Mobility Shift Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ incubated with ^{32}P-labeled ssDNA on a 13% non-denaturing polyacrylamide gel visualized by phosphorimaging.

***at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ can bind to dsDNA**

To test the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to bind double-stranded DNA, an EMSA was performed by incubating radiolabeled duplex DNA with purified *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ at increasing concentrations (**Figure 3**). Without the presence of protein (lane 1), no protein-dsDNA complex was formed. The unbound dsDNA appeared near the bottom of the gel. As the concentration of *at*Dmc1$_{WT}$ was increased, a protein-dsDNA complex was formed (lanes 2-5). As with the ssDNA EMSA, the larger size of this complex causes it to not migrate as quickly on a polyacrylamide gel. Similarly, in the absence of protein, the unbound dsDNA did not form a protein-ssDNA complex (lane 6). As the concentration of *at*Dmc1$_{A200V}$ increased, a protein-dsDNA complex was also formed (lanes 7-10). Both *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ can bind to dsDNA, but further work also needs to be done to optimize these EMSA conditions.

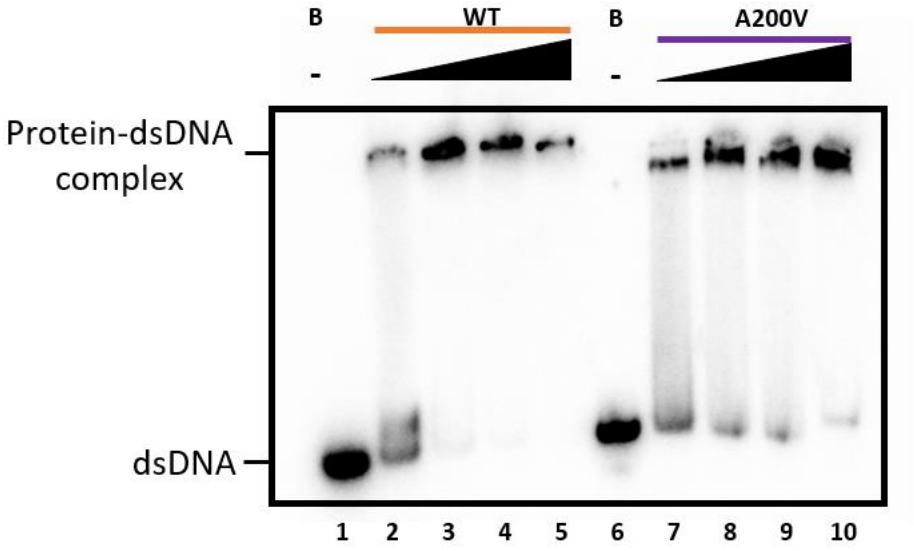

Figure 3. Electrophoretic Mobility Shift Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ incubated with radiolabeled duplex DNA on a 13% non-denaturing polyacrylamide gel visualized by phosphorimaging.

hDMC1$_{WT}$ can Perform D-Loop Activity in the Absence of Accessory Factors

To model the activity of human DMC1 protein catalyzing the formation of a D-Loop *in vitro*, this protein was incubated with ^{32}P-labeled ssDNA that is complementary to unlabeled supercoiled dsDNA. If the DMC1 protein can catalyze the formation of a D-Loop, this larger structure will migrate to a higher position on a polyacrylamide gel after electrophoresis than the radiolabeled ssDNA will migrate by itself. As seen in **Figure 4**, without the addition of protein, no D-Loop activity is found (lane 1). After 0.5-minute incubation, there is weak presence of D-Loop formation (lane 2). After 3 minutes of incubation, a stronger signal is found, indicating that hDMC1 is able to catalyze D-Loop formation without accessory factors (lane 3).

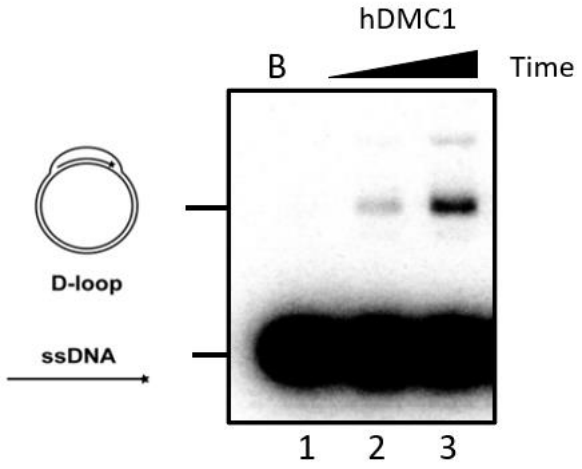

Figure 4. Displacement Loop Activity Time Course Assay of hDMC1$_{WT}$

***at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ are Unable to Catalyze D-Loop Formation Without Accessory Factors**

Following the finding that hDMC1 can catalyze D-Loop formation without accessory factors, and that *at*Dmc1 can physically interact with ssDNA and dsDNA, a similar time-course D-Loop activity assay was performed with wild-type and mutant *at*Dmc1 proteins (**Figure 5)**. Without the presence of protein, no D-Loop formation was observed. At both the 0.5, 3, and 8 minute time points, no D-Loop formation was observed with addition of *at*Dmc1$_{WT}$ (lanes 2-4). Similarly, no D-Loop formation was observed with addition of *at*Dmc1$_{A200V}$ at identical time-points (lanes 5-7). This finding indicates that, unlike hDMC1, *at*Dmc1 cannot perform D-Loop activity by itself.

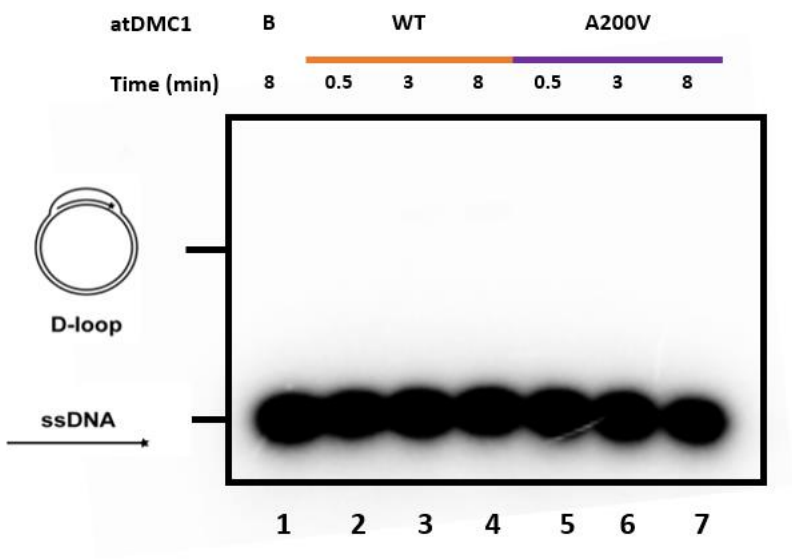

Figure 5. Displacement Loop Activity Time Course Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$

$CaCl_2$ Stimulates D-Loop Formation of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$

Calcium ions have been shown to stimulate D-Loop formation in humans, so the addition of calcium to the D-Loop activity assay was the next step for this experiment. A similar time course was performed as in **Figure 5**, but calcium was incubated with the reaction product prior to incubation with the unlabeled supercoiled dsDNA (**Figure 6**). Without the presence of protein (lane 1), no D-loop activity was recorded. With increasing time (lanes 2-4), D-Loop formation was stimulated by the presence of calcium ions for *at*Dmc1$_{WT.}$ Upon omission of calcium, no D-Loop formation was seen for *at*Dmc1$_{WT}$ (lane 5). Similarly, D-Loop formation for *at*Dmc1$_{A200V}$ was stimulated by the presence of calcium (lanes 6-8), and no D-Loop formation was observed when calcium was omitted (lane 9). *at*Dmc1$_{A200V}$ seems to be severely attenuated in D-Loop formation when compared to *at*Dmc1$_{WT}$ (compare lanes 4 and 8). These findings suggest that calcium is a necessary accessory factor for *at*Dmc1. They also suggest that the mutant *at*Dmc1is compromised in its ability to perform D-Loop.

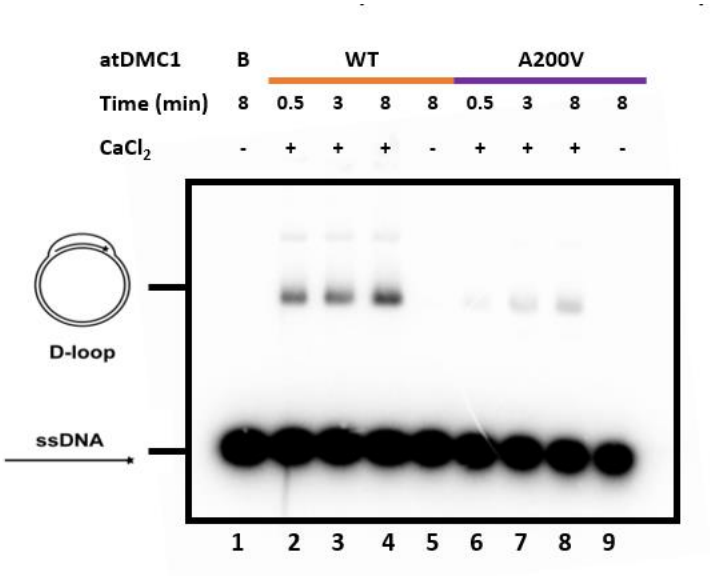

Figure 6. Displacement Loop Activity Time Course Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ with $CaCl_2$ Addition

H2M1 Stimulates D-Loop Formation of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$

H2M1 has also been found to stimulate D-Loop activity in hDMC1$_{WT.}$ To determine the impact of H2M1 on D-Loop formation by *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$, an end point D-Loop assay was performed with addition of H2M1 (**Figure 7**). Additionally, the impact of calcium and H2M1 was investigated using this activity assay. Without the addition of any protein, no D-Loop activity was observed (lane 1). Upon addition of increasing concentrations of *at*Dmc1$_{WT}$ (0.38, 0.64, 1.02, 1.36, 1.70, 2.04 µg), D-Loop formation increased (lanes 2-7). Upon omission of H2M1, no D-Loop activity was seen (lane 8). Upon addition of calcium, an intense band of D-Loop activity was observed (lane 9). Upon addition of calcium and H2M1, a slightly less intense but still dark band was formed (lane 10). The *at*Dmc1$_{A200V}$ mutant was similarly tested for D-Loop activity upon addition of both calcium and H2M1. The D-Loop product appeared as a dark band, comparable with that of the *at*Dmc1$_{WT}$ with addition of calcium and H2M1 added (lane 11). The ideal amount of *at*Dmc1$_{WT}$ for D-Loop formation was determined to be 1.02 µg (lane

5), as no significant increase was observed upon increased concentrations. H2M1 seems to be an accessory factor for stimulating *at*Dmc1$_{WT}$ D-Loop formation, however calcium seems to enhance the formation on its own to a greater degree than H2M1 on its own (compare lanes 7 and 9). Additionally, the combined stimulatory effects of H2M1 and calcium together appear to be greater than H2M1 on its own (compare lanes 7 and 10), but slightly less than calcium on its own (compare lanes 9 and 10). These findings necessitated more research into the interaction of H2M1 and calcium on D-Loop formation by *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$.

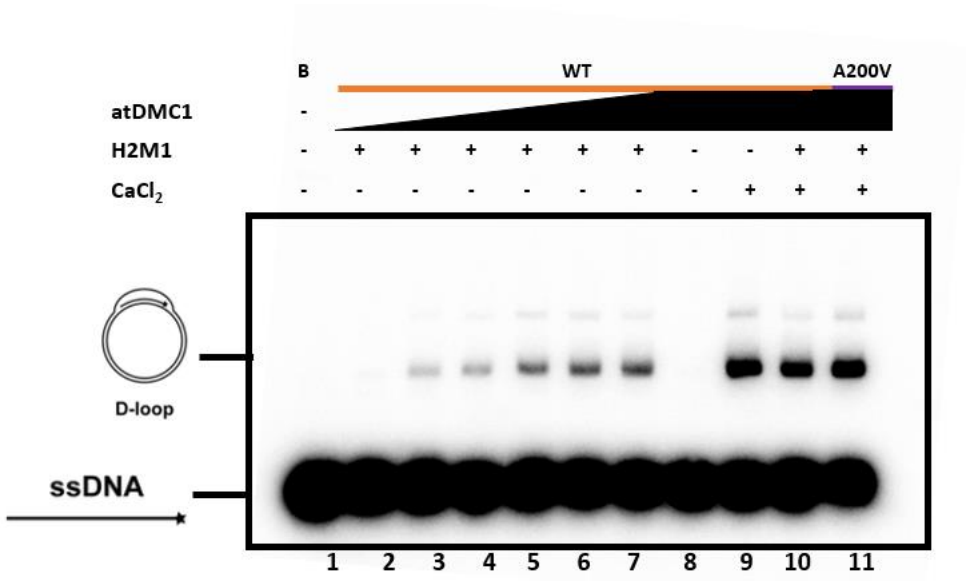

Figure 7. Displacement Loop Activity End Point Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ with H2M1 Addition

Impact of H2M1 and $CaCl_2$ on D-Loop Formation of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$

To further determine the interaction of H2M1 and calcium on D-Loop formation by *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$, a time-point D-Loop activity assay was performed (**Figure 9**). Without addition of protein, no D-Loop activity was observed (lane 1). At increasing time (0.5,

3, 8 minutes), with addition of *at*Dmc1$_{WT}$, there was a marked increase in D-Loop formation with both H2M1 and calcium present (lanes 2-4). The reaction seems to reach a peak around 3 minutes, as there is not a noticeable increase in D-Loop activity between 3 and 8 minutes. With H2M1 alone, there is a decrease in D-Loop activity (lane 5). With calcium alone, there is a significant increase in D-Loop activity when compared to H2M1 and calcium together (lane 6). With only protein present, there is very little D-Loop activity for *at*Dmc1$_{WT}$ (lane 7). This indicates that while H2M1 and calcium both have stimulatory effects on D-Loop formation by *at*Dmc1$_{WT}$, their combined impact is greater than H2M1 stimulation alone, but less than calcium stimulation alone (compare lanes 3, 4, and 5). For *at*Dmc1$_{A200V}$, the addition of H2M1 and calcium at increasing times provides a stimulatory impact (lanes 8-10). The reaction seems to be done by the 0.5-minute mark, as there is little observed increase in D-Loop formation after this time. With H2M1 alone, there is no D-Loop activity observed (lane 11). With calcium alone, there is a stimulation of D-Loop activity, but it is less than that of H2M1 and calcium stimulation together for *at*Dmc1$_{A200V}$ (lane 12). With only *at*Dmc1$_{A200V}$ present, there is no observed D-Loop activity (lane 13). Taken together, these results indicate that *at*Dmc1$_{A200V}$ outperforms D-Loop activity in the presence of both H2M1 and calcium when compared to *at*Dmc1$_{WT}$ (compare lanes 2-4 and 8-10). *at*Dmc1$_{WT}$ outperforms *at*Dmc1$_{A200V}$ with only H2M1 present (compare lanes 5 and 11). Additionally, *at*Dmc1$_{WT}$ outperforms *at*Dmc1$_{A200V}$ with only calcium is present (compare lanes 6 and 12). Interestingly, the combined stimulation of H2M1 and calcium is less than that of calcium alone for *at*Dmc1$_{WT}$ (compare lanes 4 and 6), but the combined stimulation is greater than that of calcium alone for *at*Dmc1$_{A200V}$ (compare lanes 10 and 12).

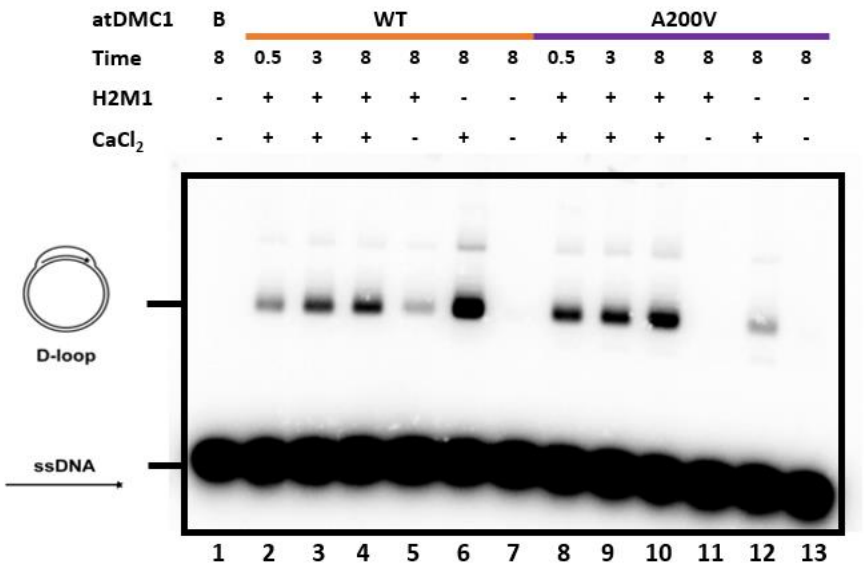

Figure 8. Displacement Loop Activity Time Course Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ with H2M1 and Calcium Addition

Calcium Enhances *at*Dmc1$_{WT}$ Strand Exchange Activity

Based of the results of the D-Loop activity assays, calcium was added to determine the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to perform strand exchange. An unlabeled ss90mer was incubated with *at*Dmc1$_{WT}$ and a complementary radiolabeled ds40mer as described in Methods. If *at*Dmc1 can catalyze strand exchange, then a larger tail-end overhand product will be formed that will migrate to a higher position on a non-denaturing polyacrylamide gel after electrophoresis. The radiolabeled ds40mer that is unable to anneal to the ss90mer will at a lower position on the gel due to its smaller size. *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ were incubated with these oligonucleotides and calcium addition to determine if they could perform strand exchange (**Figure 9**). With no protein present, no tail-end overhand product was observed (lane 1). Upon increasing time of incubation with *at*Dmc1$_{WT}$, increased tail-end overhang product was observed (lanes 2-5). Interestingly, no tail-end overhang product was observed upon incubation with

*at*Dmc1$_{A200V}$ at any time point, indicating that the mutant is severely attenuated in its ability to perform strand exchange, even in the presence of calcium (lanes 6-9).

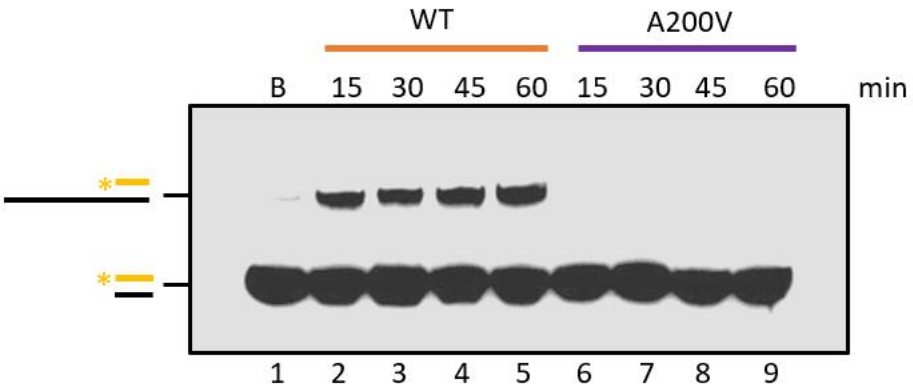

Figure 9. Single Strand Exchange Activity Time Course Assay of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ with Calcium Addition

Discussion

The *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ proteins were able to be successfully purified, which gives aid to future research into *at*Dmc1 mutants to follow in the purification scheme outlined in this paper. The ability of DMC1 to function properly is paramount for proper repair of DNA double strand breaks through homology directed repair. Following a DNA double-strand break, 5' recession occurs to generate 3' ssDNA overhands occur. These 3' overhands serve as sites for nucleation by DNA recombinases (such as DMC1) to form a nucleoprotein filament (12). This nucleoprotein filament then searches for homology in the homologous sister chromatid. Once found, the nucleoprotein filament catalyzes strand invasion, displacing the complementary strand to form a displacement loop. This displacement loop is then used to restore the damaged DNA,

using the homologous region. Due to this function, the ability of DMC1 to bind to both single and double stranded DNA is of great importance. One of the first objectives in this paper was to determine the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to bind to both single and double stranded DNA through the use of an electrophoretic mobility shift assay. Based off of the results of **Figure 2** and **Figure 3**, both *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ are able to bind to both single stranded and double stranded DNA. Thus, DNA binding ability is likely not the reason for the sterility phenotype observed in *at*Dmc1$_{A200V}$ mutants.

Knowing that both *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ can bind DNA, the next step in determining the reason behind the sterility phenotype was in the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to catalyze D-Loop formation. Human DMC1 is known to be able to catalyze D-Loop formation in the absence of accessory factors (**Figure 4**), but *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ are unable to do so (**Figure 5**). Knowing that human DMC1 is stimulated by the presence of calcium ions, *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ were tested for their ability to catalyze D-Loop formation under the addition of calcium. As seen in **Figure 6**, both *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ were able to catalyze D-Loop formation in the presence of calcium, albeit the mutant was severely attenuated in their ability to do so. It is proposed that calcium stimulates a conformation change by interacting with DMC1, and this proposed model may be the reason for enhanced D-Loop activity under these conditions. Similarly, H2M1 is thought to induce a conformational change in DMC1 to allow it to conduct D-Loop activity. Through an end-point D-Loop activity assay, the ability of H2M1 to stimulate *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ D-Loop activity was observed. With the addition of H2M1, *at*Dmc1$_{WT}$ was able to increase its D-Loop activity, although to a lesser degree than the addition of calcium (**Figure 7**). With addition of both H2M1 and calcium, *at*Dmc1$_{WT}$ was stimulated above levels for H2M1 by itself, but less than for

calcium by itself. Interestingly, *at*Dmc1$_{A200V}$ D-Loop activity was not stimulated by H2M1 by itself, was stimulated by calcium by itself, and was stimulated to a greater degree by the addition of both H2M1 and calcium (**Figure 8**). Some interaction between *at*Dmc1$_{WT}$, H2M1, and calcium changes the conformation of the Dmc1 protein so that it performs D-Loop to a greater degree when only calcium is present than when H2M1 and calcium are present. However, *at*Dmc1$_{A200V}$ performs D-Loop to a greater degree when H2M1 and calcium are present than when calcium or H2M1 alone are present. Bioinformatic modeling of the structure of *at*Dmc1$_{WT}$ or *at*Dmc1$_{A200V}$, with addition of calcium and H2M1 could provide further insight into this phenomenon.

Similarly, *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ differ in their ability to perform strand exchange in the presence of calcium (**Figure 9**). Through the use of a strand-exchange time point assay, it was determined that *at*Dmc1$_{WT}$ can perform strand exchange under the addition of calcium, while *at*Dmc1$_{A200V}$ is severely attenuated in its ability to do so. A future area of this research would be to add H2M1 to the assay to determine if H2M1, or H2M1 and calcium together can overcome this inhibition.

Further future directions for this research involve determining if *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ have the ability to utilize ATP to form a filament on DNA and search for homology in the homologous sister chromatid through an ATP hydrolysis assay. Additionally, the ability of *at*Dmc1$_{WT}$ and *at*Dmc1$_{A200V}$ to form a filament on DNA should be investigated using a nuclease protection assay. As always, these results are *in vitro* and may not be able to be directly generalized to results *in vivo*. Despite this, this implications for these findings in the impact of DMC1 protein in sterility, and the ability of recombinases to aid in the resolution of DSB repairs may be useful in the creation of fertility treatments and in treating cancer (12).

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