UNIVERSITY OF CALIFORNIA SAN DIEGO

In vitro reconstitution of DNA unwinding during replication in human mitochondria

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by

Xing Quan

Committee in charge:

Professor Tatiana Mishanina, Chair
Professor Galia Debelouchina
Professor Mark Herzik

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The thesis of Xing Quan is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THESIS

In vitro reconstitution of DNA unwinding during replication in human mitochondria

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Professor Tatiana Mishanina, Chair

Mitochondrial DNA (mtDNA) plays an important role in mitochondria and its maintenance is critical for sustaining aerobic respiration in cells. However, the details of mtDNA replication mechanism in human mitochondria are still unclear. Three existing models of this process have been put forward to date, which are strand-displacement model, bootlace model and strand-coupled DNA replication model but several contradictions exist among these models. To study the mechanism of mtDNA replication in humans, two proteins, TWINKLE helicase and mitochondrial single-stranded DNA binding protein (mtSSB), that participate in this process were purified and their activity was tested using fluorescein amidite (FAM)

labeled DNA and radiolabeled DNA. With these two proteins, part of the mtDNA replication system was rebuilt *in vitro*. It is found that mtSSB promotes the activity of TWINKLE helicase by protein-protein interaction instead of binding to unwound DNA as claimed in the strand-displacement model. Based on size exclusion chromatography and SDS-PAGE analysis, it is suggested that mtSSB and TWINKLE helicase form a complex during the unwinding process. This experiment builds a platform for further structural study of the mtDNA replication mechanism and protein-protein interaction between mtSSB and TWINKLE helicase.

Introduction

Mitochondria are double membrane-bound organelles in eukaryotic cells with many critical functions, such as fatty acid oxidation for acetyl-CoA production, and biosynthesis of heme, iron-sulfur clusters and nucleotides, to name a few. This organelle was first observed in the 1840s and the term 'mitochondria' was coined by Carl Benda in 1898.[1][2] Mitochondria are where the aerobic respiration takes place to produce most of the ATP in a cell, which is why mitochondria are nicknamed 'the power house of the cell'.[3] A unique feature of mitochondria is that they contain their own multicopy genome, which is transcribed and replicated by a protein system completely different from its nuclear counterparts. [4]

Human mitochondrial DNA (mtDNA) is a circular molecule that contains 16569 base pairs (Fig. 1). The complete sequence of mtDNA was first determined and published by Stephen Anderson in 1981.[5] The copy numbers of mtDNA per mitochondria range from dozens to thousands in different cell lines. [6] One strand of mtDNA contains more guanines than the other strand, making it possible to separate the two strands by density centrifugation in alkaline $CsCl_2$. Based on the density, the guanine-rich strand is referred to as the heavy (H) strand, and the other strand is called light (L) strand. [7]

Human mtDNA is a highly compact molecule that encodes 13 messenger RNAs (mRNAs), 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs), without any intronic structure in protein-coding genes. The 13 proteins translated from these mRNAs form subunits of Oxidative Phosphorylation (OXPHOS) complexes I, III, IV, and V (Fig. 1). [5] The mtDNA contains a long non-coding region (NCR) of approximately 1 kb, which controls transcription and replication of mtDNA. Transcription of mtDNA starts at three promoters in this region, which are light-strand promoter (LSP) and heavy strand promoters (HSP1 and HSP2), and the origin of heavy strand DNA replication (O_H) is also in this region. [8] The origin of light strand

DNA replication (O_L) is located outside of NCR, approximately 11kb downstream of O_H .[9][10]

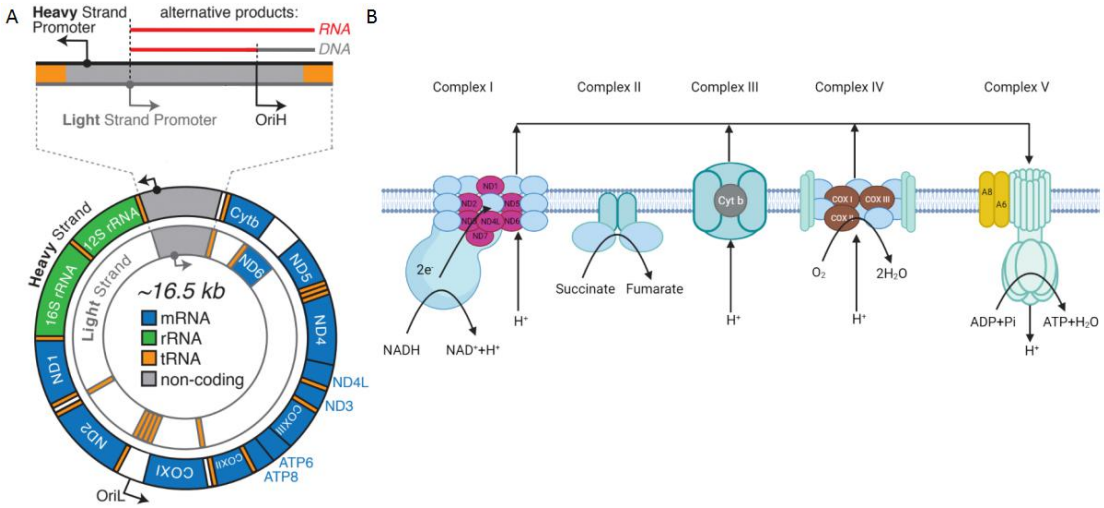

Figure 1: (**A**) Schematic presentation of human mtDNA. Gene-coding regions for mRNA, rRNA and tRNA are shown as blue, green and orange,respectively. The major non-coding region (NCR) is shown as gray. The two transcription promoters, light-strand promoter (LSP) and heavy-strand promoter (HSP), are located in the NCR. LSP is responsible for the transcription of 1 mRNA and 8 tRNAs. HSP is responsible for the transcription of 12 mRNAs, 14 tRNAs and 2 rRNAs. The initiation site for heavy-strand replication (OriH,O_H) is also located in the NCR, while the initiation site for light-strand (OriL,O_L) is located outside of NCR, about 2/3 of the way down from the LSP transcription site. (**B**) Schematic of Oxidative Phosphorylation (OXPHOS) at the inner mitochondrial membrane. Protein subunits encoded by mtDNA are highlighted in dark blue. ND1, 2, 3, 4, 4L and 5 (purple) are subunits of OXPHOS complex I. Cyt b (orange) is subunit of complex III. COX I, II and III (green) are subunits of complex IV. ATP 6 and ATP 8 (yellow) are subunits of complex V.

Because the OXPHOS system relies on proteins encoded by mtDNA, maintenance of mtDNA is essential for cells. Mutations on mtDNA can lead to serious disease. For example, point mutations in the mitochondrial ATPase 6 gene lead to Leigh's syndrome, which is a serious neurological disorder disease.[11][12] Mutations of lysine tRNA cause Myoclonic epilepsy with ragged-red fibers (MERRF), which is a neuromuscular disorder that affects many parts of the body.[13] Thus, it is important for mtDNA to have a faithful replication system, to avoid introduction of such deleterious mutations. The mtDNA replication system contains several mitochondria-specific proteins including TWINKLE DNA helicase, DNA polymerase γ (POL γ),

mitochondrial RNA polymerase (POLRMT) and mitochondrial single-stranded DNA binding protein(mtSSB). [14] Other proteins including RNase H1, DNA ligase III and topoisomerase 3α also participate in mtDNA replication. The knockout of these proteins leads to disfunction of mitochondria. [15][16][17][18]

Several models for how mtDNA is replicated have been put forward over the years. The earliest mtDNA replication model was the **strand-displacement model** presented in 1972 by Robberson and Vinograd.[19] In this model, DNA replication is continuous on both L-strand and H-strand but is initiated at two different sites on the two strands (Fig. 2). Heavy strand replication is initiated at O_H site by POLRMT synthesizing a short RNA primer. Using this RNA primer as a starting substrate, POL γ begins synthesis of the nascent heavy DNA strand, with the light strand as a template. Because POL γ can only process single-stranded DNA (ssDNA) as a template for copying DNA, this process relies on TWINKLE DNA helicase unwinding the double-stranded DNA (dsDNA) to generate ssDNA for POL γ. The other parental strand (also called the lagging strand) is coated by mtSSB in the meantime, protecting it from the attack by nucleases and non-specific transcription initiation by POLRMT. After the replication fork passes through the O_L site, the single-stranded parental heavy strand DNA is proposed to form a stem loop structure, thereby blocking mtSSB binding to DNA in this region. Instead, POLRMT will synthesize a short RNA primer using the exposed ssDNA as a template. Then POL γ will start replication of the light strand using this primer. In this model, both the H-strand and L-strand replication are continuous and unidirectional; there are no Okazaki fragments involved in this process. This DNA replication mechanism is classified as strand-asynchronous replication. The major evidence that supports this model was published by Van Tuyle in 1985, where mtSSB coating one parental strand of a replicating mtDNA was observed by electron microscopy. [20]

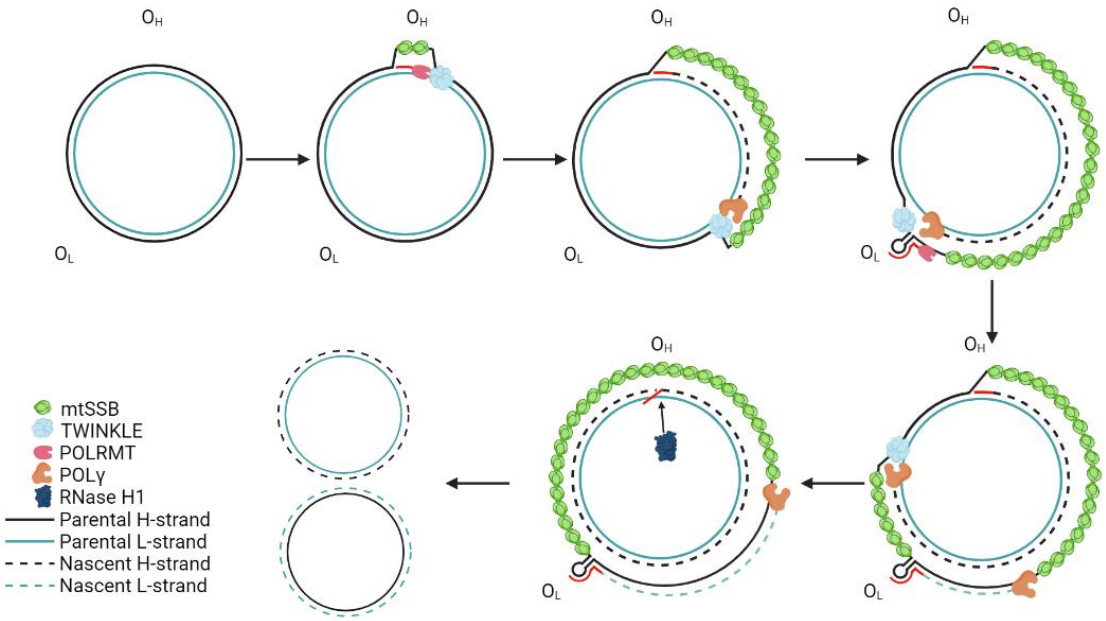

Figure 2: Schematic diagram of strand-displacement model. In this model, the mtDNA replication is initiated at O_H by POLRMT synthesize a short RNA primer. TWINKLE helicase keep unwinds the mtDNA and POLγ generates the nascent strand unidirectionally using the parental light-strand as a template. The other strand was covered by mtSSB. On the O_L site, a stem loop structure is formed to prevent mtSSB binding and the replication of light strand is initiated.

In the beginning of the 21st century, 2D-agarose gel electrophoresis (2D-AGE) has been used to examine the mtDNA replication mechanism. Researchers used restriction enzymes to digest the replicating mtDNA isolated from cells and separate the DNA fragments with 2D-AGE. Fragments with different structures will form different migration pattern on the gel (Fig. 3G). The characteristic pattern of strand-displacement replication is a slow moving Y-like arc (SMY arc) because one strand of the replication bubble is protected by mtSSB and thus cannot be cleaved by a restriction enzyme (Fig. 3).[21] Interestingly, when RNase H, an enzyme that can digest DNA-RNA hybrid, was added, the SMY disappeared from the gel.[22] This result is inconsistent with the strand-displacement model's proposal that mtSSB coats the unwound parental DNA. Instead, it suggests that the parental strand is protected by formation of a DNA-RNA hybrid.

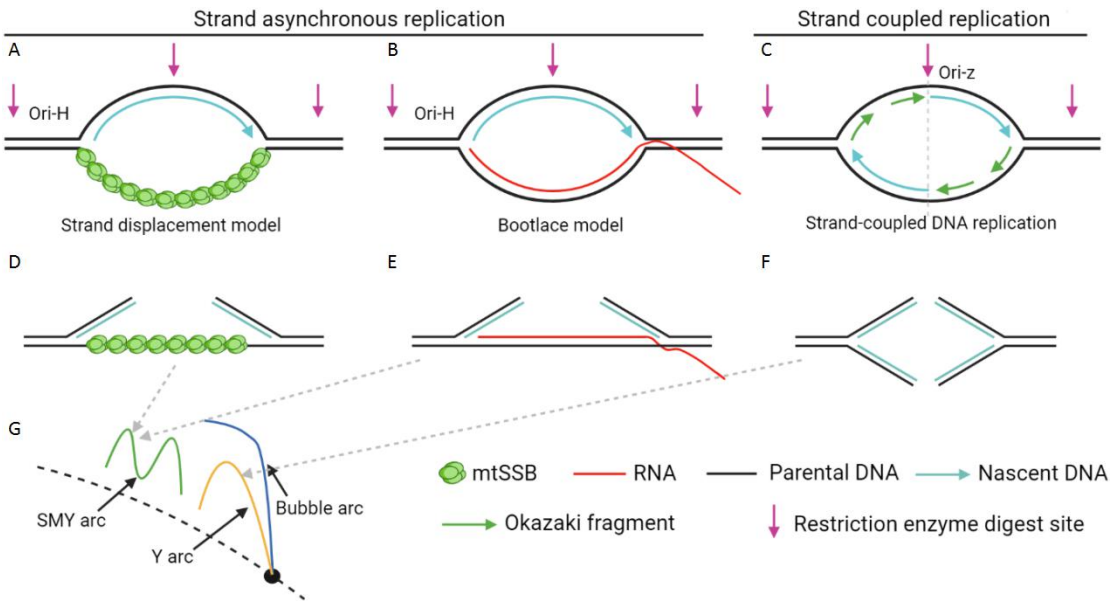

Figure 3: (**A**, **B**, **C**) Models of mtDNA replication. In a strand-displacement model (**A**), the ssDNA is coated by mtSSB. In a bootlace model (**B**), the ssDNA forms a hybrid with an RNA transcript. In a strand coupled DNA replication (**C**), the mechanism is similar to the DNA replication in nucleus, with lagging strand replication occurring simultaneously with the leading strand via Okazaki fragment formation. (**D**, **E**, **F**) Restriction digestion product of replication intermediates. A restriction enzyme only cuts within double-stranded DNA regions. (**G**) Different digestions products from different pattern in 2D-AGE experiment.

To explain the 2D-AGE result above, a **bootlace model** was proposed.[23] In this model, the initiation and elongation mechanisms are similar to strand-displacement model, but instead of mtSSB coating the lagging strand, an RNA transcript is constantly hybridized to the lagging strand. The lagging-strand replication is initiated at O_L site and the RNA transcript is gradually replaced by the nascent light strand. This DNA replication mechanism is also classified as strand-asynchronous replication. The major problem for this model is that it does not explain the initiation mechanism at O_L site. Besides, it does not explain how the DNA-RNA hybrid survives from the attack of RNase H1, a mitochondrial enzyme that can degrade the hybrid.[24]

Another model, **strand-coupled DNA replication** (SCD replication) model was also raised in early 2000s because a Y arc was observed in 2D-AGE experiment (Fig. 3F), which indicates a bidirectional replication mechanism.[25] In this model, the

replication initiates over a broad zone of several kilobases (Ori-z)[26] and the replication is synchronous and bidirectional. Like the DNA replication mechanism in the nucleus, POL γ continuously replicates the heavy strand from 5' to 3'. On the other strand, POL γ is primed by many short RNA primers and generates multiple Okazaki fragments.[27] DNA ligase then ligates these fragments and creates a nascent light strand. This model is quite nascent and lacks solid experiment support because many of the enzymes involved are still unidentified.

It is still unclear which kind of replication model is the predominant mechanism that mitochondria use to reproduce its DNA. Some experiments suggest that mitochondria may change the DNA replication mechanism under different conditions. For example, Cluett et al. had found that when there is little transcription happening in the mitochondria, the major replication mechanism will turn to SCD replication.[28] Besides, there is no study on the mechanism of mtDNA replication initiation. Since the regular function of mitochondria relies on mtDNA, it is important to understand the replication mechanism of mtDNA. Over the course of my thesis work, I have rebuilt a part of the mtDNA replication system *in vitro* and studied the activities and potential interaction between the proteins in this system.

Results

Purified recombinant TIWKNLE helicase possesses DNA unwinding activity

To study human mtDNA replication mechanism, I purified two proteins that participate in this process: TWINKLE helicase and mtSSB. TWINKLE helicase belongs to the SF4 helicase family and has both DNA annealing and unwinding activities.[29] [30] TWINKLE helicase can form multiple oligomeric states including dimer, trimer, tetramer, pentamer and hexamer. Atomic force microscopy (AFM) shows that TWINKLE forms a heterogeneous mixture of oligomers in the absence of DNA. When functioning on DNA, however, the lower-order oligomers of TWINKLE self-assemble into a closed-ring hexamer that binds DNA substrate. [31]

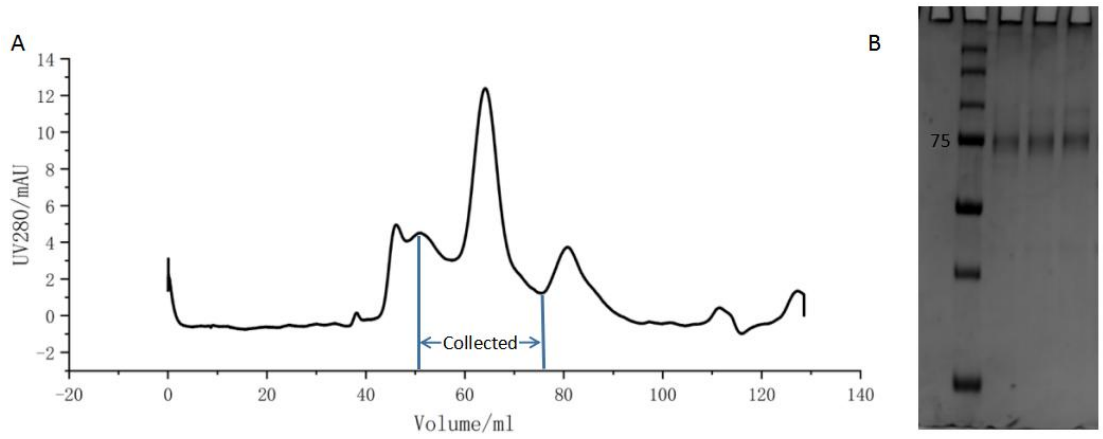

Figure 4. (**A**) Size exclusion chromatogram of the final step in TWINKLE helicase purification. The collected fractions are shown between the blue lines. (**B**) SDS-PAGE result of the fractions collected from size exclusion chromatography. The molecular weight of recombinant TWINKLE helicase monomer is 72.13 kDa.

I tried several different constructs for recombinant expression of TWINKLE helicase and found that this protein is sensitive to N-terminus tagging. N-terminus tag leads to aggregation and disfunction of this protein. In the final construct, I put a His_6 tag at the C-terminus of TWINKLE. This recombinant protein was expressed in *E.coli* and purified with Ni^{2+} affinity chromatography. I then used ion exchange chromatography to remove most of the negatively charged contaminating proteins.

Because TWINKLE helicase is a DNA-binding protein, I used heparin column for further purification and to outcompete any genomic bacterial DNA bound to TWINKLE. The last step of purification was size exclusion chromatography (SEC) that removed TWINKLE aggregates and remaining contaminating proteins. Because TWINKLE forms a heterogeneous mixture of oligomeric states, I collected and pooled several peaks of the SEC eluent, corresponding to the expected sizes of these oligomers (Fig. 4).

TWINKLE helicase cannot directly bind to dsDNA, it requires a 5' single-stranded overhang on the dsDNA to start the unwinding process. *In vivo* such stretch of single-stranded DNA is likely produced by POLRMT as part of transcription process.[32] In order to test the DNA unwinding activity of TWINKLE, I used a 'fork' DNA (Fig. 5A) that contains a 35-nt single-stranded 5' overhang, so TWINKLE can assemble on it and begin unwinding dsDNA downstream of the overhang. On the native PAGE gel, there is a clear shift from fork DNA site to ssDNA site when 1 nM of TWINKLE was added. When 0.2 nM of TWINKLE was added, there was also a shift on the gel although with a much slower kinetics. However, when fork DNA alone was incubated in the reaction buffer for 2 hours, there was no band shift on the gel (Fig. 5B, C). This result shows that the TWINKLE helicase I purified has DNA unwinding activity. The calculated unwinding rate is 4.9×10^{-5} nM/min, which is close to the TWINKLE activity reported before (4.4×10^{-5} nM/min).[33]

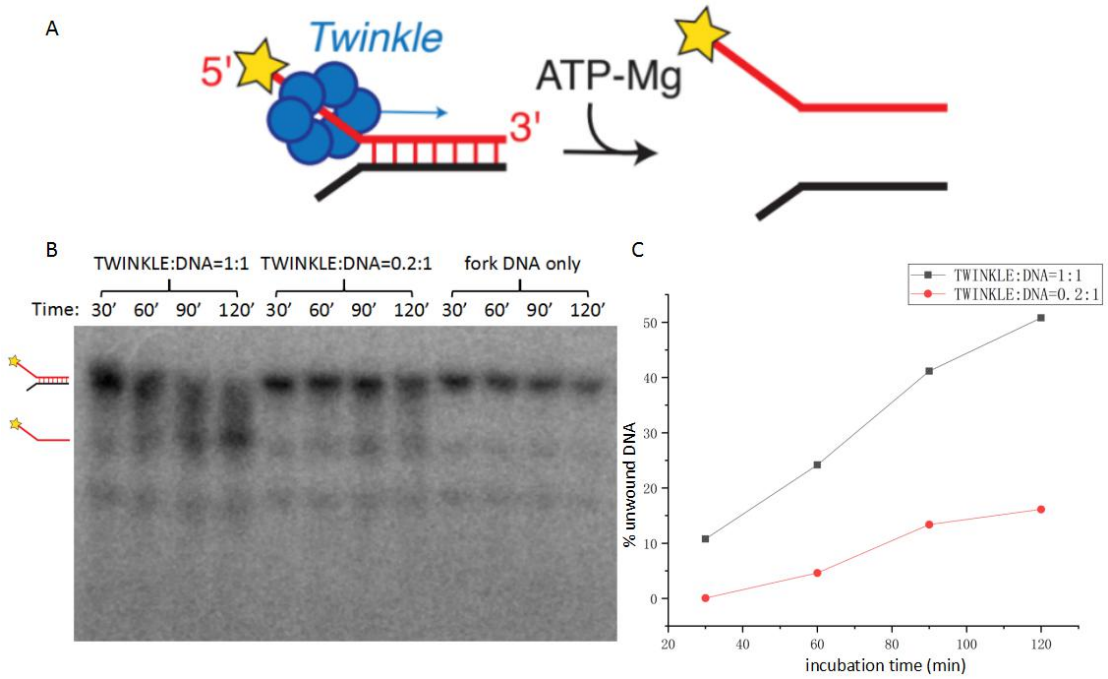

Figure 5: TWINKLE activity assay result. (**A**) 5'-labeled fork DNA was designed for the unwinding assay. (**B**) 1, 0.2 and 0 nM of TWINKLE helicase was incubated with 1nM of fork DNA for 30, 60 90, 120 minutes. Gel result shows that TWINKLE has unwinding activity. (**C**) Quantification of the native PAGE gel.

Purified recombinant human mtSSB binds ssDNA

Human mtSSB is a small protein with a molecular weight of 15.2 kDa. It contains an oligonucleotide/oligosaccharide-binding (OB)-fold and a protein domain that is responsible for interacting with other proteins.[34] MtSSB forms a homotetramer that binds ssDNA via two different proposed models, depending on the presence of magnesium ions in its environment. In the presence of Mg^{2+}, ssDNA will wrap around all four mtSSB subunits, with each tetramer binding ~60 nucleotides while in the absence of Mg^{2+}, ssDNA will only wrap around two subunits and each tetramer will only bind 30 nucleotides. [35]

I introduced a His_6-GST tag at the N-terminus of mature form mtSSB(17-148, lacking mitochondrial-targeting sequence) to increase the solubility and help mtSSB to fold correctly during recombinant expression. The recombinant protein was expressed in *E.coli* and purified with affinity chromatography. Because mtSSB is a DNA-binding protein like TWINKLE helicase, I used heparin affinity purification strategy to purify it. Size exclusion chromatography was also used for final

purification (Fig 6).

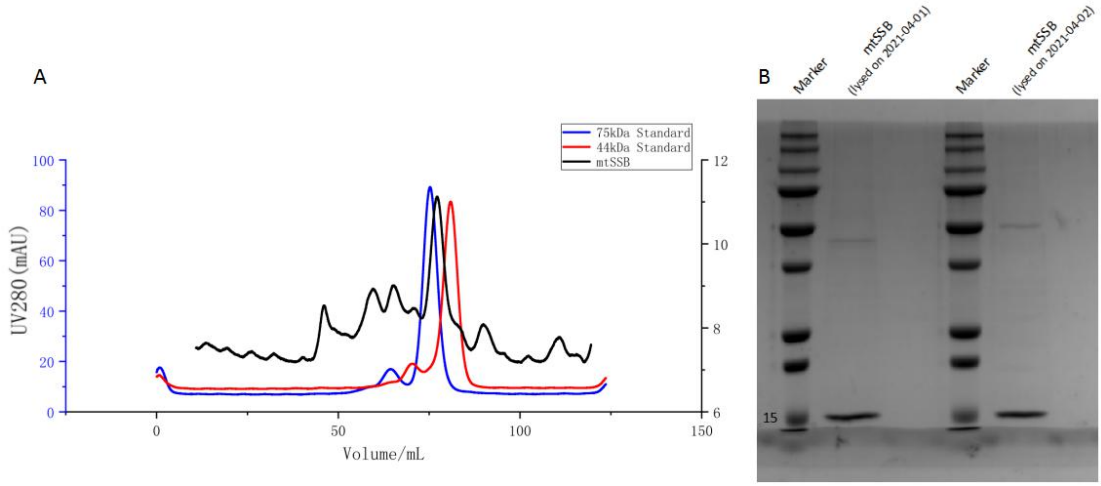

Figure 6: (**A**) Size exclusion chromatography graph in mtSSB purification. The molecular weight of mtSSB tetramer is 60.8 kDa. 75kDa standard is shown in blue line and 44 kDa is shown in red line. (**B**) SDS-PAGE result of the fractions collected from size exclusion chromatography. The molecular weight of recombinant mtSSB monomer is 15.2 kDa.

I first used FAM-labeled ssDNA to test DNA binding activity of purified recombinant mtSSB. On the native PAGE gel, there is a clear band shift from ssDNA site (site C) to a higher position which represents ssDNA bound to mtSSB (site A)(Fig. 7C). After 10 minutes of incubation, no change in the fraction of ssDNA bound to mtSSB was observed, suggesting that an equilibrium between DNA-bound and free mtSSB has been reached in this amount of time (Fig. 7A, E). As the concentration of mtSSB increased from 2.5 mM to 10 mM, there is a significant increase in the fraction of ssDNA bound to mtSSB (Fig. 7A, D). These results together demonstrate that the mtSSB I purified has ssDNA binding activity. When tested with FAM-labeled ssDNA, I observed some DNA stuck in the wells at the top of the gel (Fig. 7A). It may be due to high protein concentration (mM) in this experiment that prevented migration of some mtSSB-bound ssDNA into the gel. To solve this problem, I repeated the assay with radiolabeled ssDNA, which thanks to the high sensitivity allows for detection of protein-bound DNA at protein concentrations as low as nM. In this assay with 2.5 nM of mtSSB, there was no DNA stuck at the top of the gel and the average fraction of ssDNA bound to mtSSB was 35% (Fig. 7B),

which is higher than for FAM-labeled DNA (23%). As a control, I also tested whether mtSSB can bind to fork DNA used for TWINKLE activity test. The result shows that mtSSB cannot bind to the 35-nt single-stranded overhang on the fork DNA and thus will not interfere with TWINKLE binding to this region in the DNA unwinding assay.

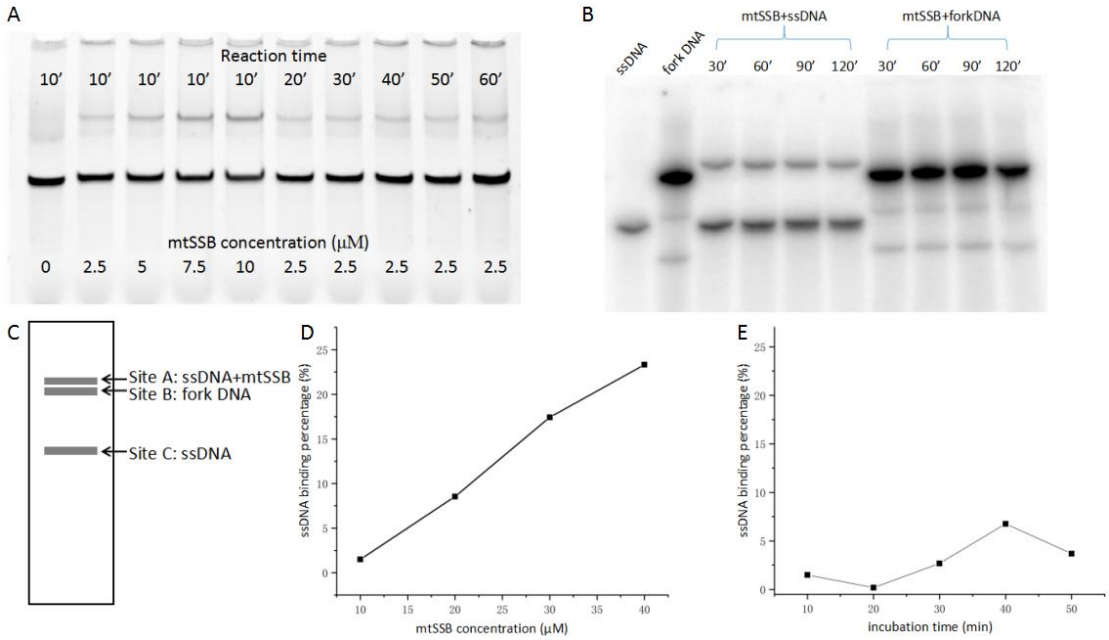

Figure 7: MtSSB activity test result. (**A**) 1 mM of 5' FAM labeled ssDNA was incubated with mtSSB for 10 to 60 minutes. The clear ban shift proves the mtSSB is active. (**B**) 1 nM of 5' radio labeled ssDNA and fork DNA was incubated with 2.5 mM of mtSSB. The fork DNA used for TWINKLE unwinding assay cannot be bound by mtSSB. (**C**) Three sites on the native PAGE gel that represent different composition of the reaction product. (**D, E**) Quantification of the native gel. The percentage of mtSSB bound DNA gets higher as the mtSSB concentration increases.

MtSSB enhances DNA unwinding activity of TWINKLE helicase.

TWINKLE helicase has been shown to perform both DNA unwinding and annealing.[36] Adding a ligand that can bind and coat the unwound ssDNA to the reaction mixture will significantly promote the unwinding activity of TWINKLE, by preventing ssDNA re-annealing. In the strand-displacement model, mtSSB is thought to be the ligand that binds ssDNA while in the bootlace model, the RNA transcript is thought to hybridize to the ssDNA (Fig. 8). Thus, I added mtSSB to the TWINKLE activity assay to test its ability to coat ssDNA product of the helicase reaction and

enhance helicase activity.

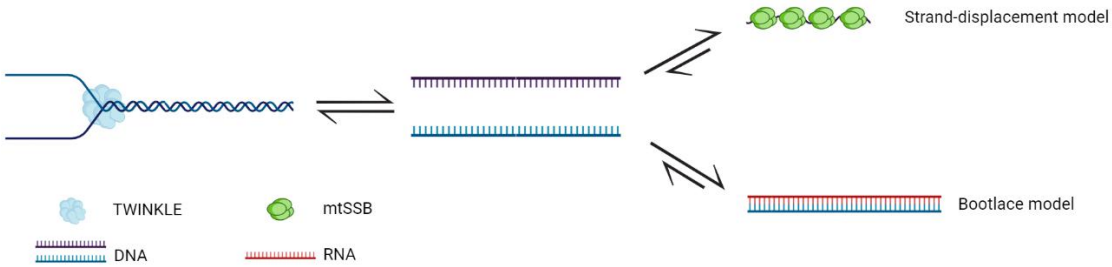

Figure 8: In the strand strand-asynchronous replication, the parental heavy strand was bound by mtSSB or RNA transcript after unwinding. Because TWINKLE has both unwinding and annealing activity, adding a ligand that can bind ssDNA should promote its unwinding activity.

When mtSSB was added to the reaction, the unwinding activity of TWINKLE significantly increased. When 40 nM of mtSSB was added to the reaction, the activity of TWINKLE was increased 45 times compared to helicase activity in the absence of mtSSB (Fig. 9B). An interesting finding here is that even though mtSSB was added to the reaction, the unwound ssDNA still migrated as free DNA, not mtSSB-bound DNA (shift to the site C instead of site A)(Fig. 7C, 9A). This result suggests that mtSSB did not stably bind to the ssDNA product of the helicase reaction but still promoted the unwinding activity of TWINKLE helicase. It is possible that mtSSB activates TWINKLE helicase by protein-protein interaction or transient interaction with unwound DNA instead of binding and coating the unwound ssDNA. These protein-protein interaction may be blocking access of mtSSB to the ssDNA. Thus, in the strand-displacement model, additional protein players may be required to free mtSSB for its assembly on the parental ssDNA.

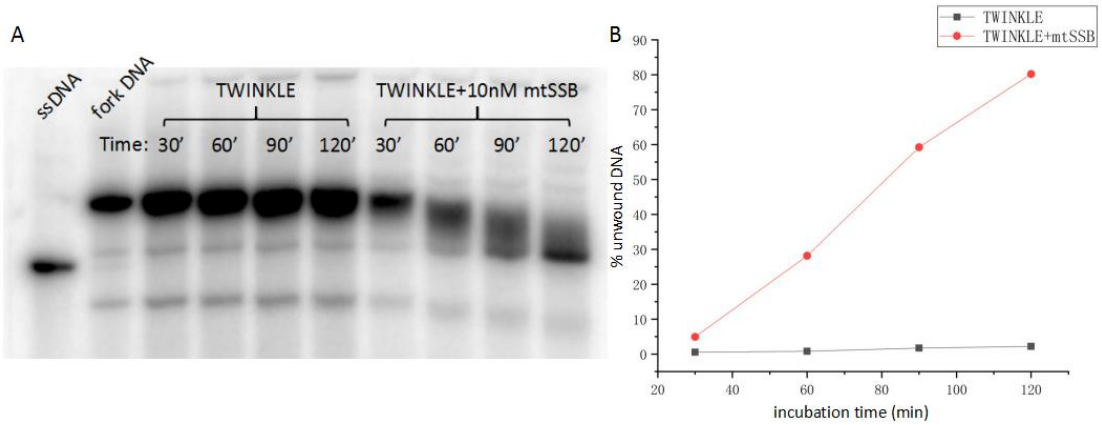

Figure 9: DNA unwinding assay with TWINKLE and SSB. (**A**) 1 nM of radio labeled fork DNA was incubated with 0.2 nM of TWINKLE. When mtSSB was added to the reaction, the activity of TWINKLE was promoted significantly. (**B**) Quantification of the Native gel. The activity of TWINKLE was promoted by 45 times when mtSSB was added.

To further investigate the DNA unwinding mechanism of TWINKLE helicase in the presence of mtSSB, I used size-exclusion chromatography to separate the components in the reaction mixture, followed by an SDS-PAGE analysis to examine the protein content in the fractions. The 280-nm UV absorbance of mtSSB and DNA is very low because mtSSB is poor in aromatic amino acids and the DNA concentration is very low in the reactions. TWINKLE helicase was eluted at 0.74 CV. When mtSSB and fork DNA were added and incubated for 2 hours, the major peak shifted to 0.64 CV, which is bigger than the TWIKNLE helicase (Fig. 10A). The SDS-PAGE result shows that both TWINKLE helicase and mtSSB is eluted in this peak, which means that these two proteins form a complex or at least will form some protein-protein interaction in the unwinding process (Fig. 10B).

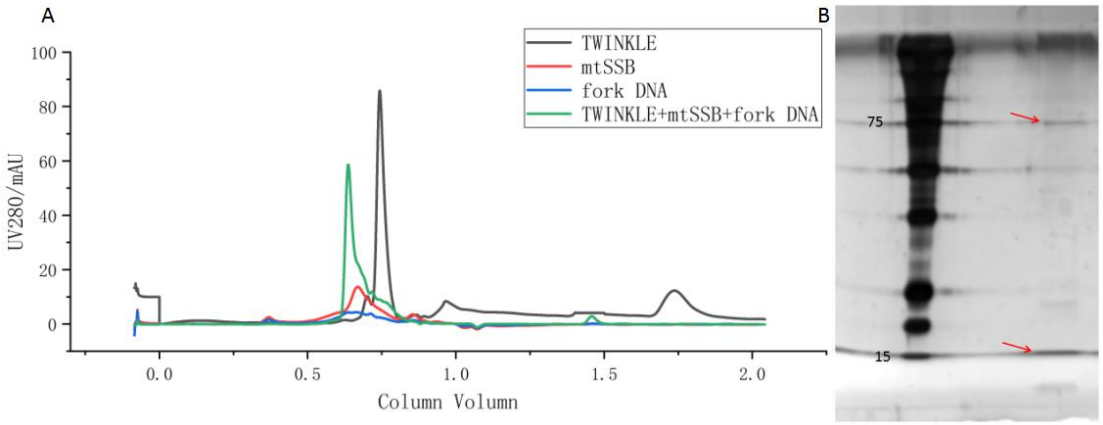

Figure 10: (**A**) The reaction mixture and all the components in the reaction was loaded on Superose 6 column separately. The concentration of each component are the same as in reaction. The peak of the reaction mixture shift to a larger position, which suggests that it forms a complex during the reaction. (**B**) Silver stained SDS-PAGE result shows that this peak includes TWINKLE helicase and mtSSB.

Discussion

To rebuild the mitochondrial DNA replication system *in vitro*, I purified two of the main proteins that participate in this process and tested their activity. The activity test result shows that the TWINKLE helicase can unwind fork DNA that contains a 5' single-stranded overhang necessary for TWINKLE loading onto DNA. The mtSSB can form a complex with ssDNA but cannot bind the fork DNA used for TWINKLE activity test. With these two proteins, I reconstituted the unwinding component of mtDNA replication and provided a platform for further study of human mtDNA replication mechanism.

I also studied the DNA unwinding mechanism of TWINKLE helicase aided by mtSSB. MtSSB can significantly promote the activity of TWINKLE helicase by about 45-fold. Interestingly, mtSSB does not seem to directly bind ssDNA product of TWINKLE unwinding reaction as claimed in the strand-displacement model[19] because I only observed ssDNA band on the native gel but there is no band that represents mtSSB bound ssDNA. Besides, the TWINKLE helicase could be blocking the binding of mtSSB to the ssDNA, which was released after unwinding.

Even though my finding does not agree directly with the strand-displacement model, it can fit within both the strand-asynchronous models. There are at least 500 mtSSB tetramers available per mtDNA molecule in the cell.[37] During the unwinding process, mtSSB could be binding to TWINKLE helicase and promoting its unwinding activity. In the strand-displacement model, other proteins in mitochondria might dislodge mtSSB from TWINKLE to free it for assembly on the unwound ssDNA to protect ssDNA from nucleases and prevent its reannealing. In the bootlace model, the activity of TWINKLE helicase is likewise promoted by mtSSB, however, mtSSB's job stops there and instead of mtSSB, an RNA transcript is hybridized to the freed ssDNA to shield it from the attack by nucleases.

In the presence of Mg^{2+}, a mtSSB tetramer can bind ~60 nucleotides of

ssDNA. The 78-nt DNA used in my unwinding assay is only sufficient for one SSB tetramer to bind. In future experiments, longer linear or circular DNA should be used to test if mtSSB can bind a longer stretch of unwound DNA generated by TWINKLE helicase. Additionally, pull down and size exclusion chromatography experiments can be performed with and without fork DNA to test if the fork DNA is required in the complex formation. Isothermal Titration Calorimetry (ITC) can also be used to determine the binding affinity between the two proteins if additional evidence for such protein complex is procured.

The mechanism of mtSSB promoting TWINKLE helicase activity is still unclear, and further experiments can be done based on my results. Since I already observed TWINKLE helicase and mtSSB forming a complex at the presence of fork DNA, this complex could be purified with size exclusion chromatography for structure determination by either X-ray crystallography or cryo-EM. Once the structure is solved, the protein-protein interface can be determined and the molecular mechanism of mtSSB promoting TWINKLE helicase activity can be explained. Hydrogen Deuterium Exchange mass spectrometry (HDX-MS) can also be used to determine the interaction interface between TWINKLE helicase and mtSSB by comparing the amide exchange rate in different parts of proteins in solution and in a complex. With the structure and protein-protein interface determined, inhibitors and enhancers of this interaction may be developed as a novel means to tune DNA replication in human mitochondria.

Material and Methods

Chemicals

Tris-base, Magnesium chloride ($MgCl_2$), Glycerol, Sodium chloride (NaCl), Dithiothreitol (DTT), Imidazole, Acrylamide, Sodium Phosphate (Na_2HPO_4) and Potassium dihydrogen phosphate (KH_2PO_4) used in the experiments were purchased from Thermo Fisher.

[γ-^{32}P] ATP used in the experiments was purchased from Perkin Elmer.

Nucleic acids

Primers and oligonucleotides used in the experiment were purchased from Eton Bioscience, Inc. (San Diego, CA). FAM-labeled oligonucleotides were bought from Integrated DNA Technologies.

Construction of recombinant proteins expression plasmids

The *E. coli*-codon optimized gene for TWINKLE helicase encoding its amino acid sequence from residue 43 to 684 was graciously provided by Dr. Smita Patel (Rutgers Robert Wood Johnson Medical School). The first 42 amino acids encode the mitochondrial targeting sequence, which is cleaved upon import of the protein into the matrix and is thus removed from this construct. A histidine (His_6) tag was added to the C-terminus of TWINKLE (43-684) by PCR. The PCR product was cleaned up using Monarch® PCR & DNA Cleanup Kit. An empty *E.coli* expression vector pET28b(+) was digested by NcoI and NotI (New England Biolabs) to generate a linearized DNA and the C-His-TWINKLE gene was transferred into this vector by Gibson assembly.

The mtSSB construct was based on Kang Li's mtSSB expression and purification protocol.[38] The first 16 amino acids are the mitochondrial targeting sequence and are removed on our cDNA. I changed their design slightly because my small-scale expression results showed that the GST tag in its mtSSB-linked

configuration cannot efficiently bind to the GST column. Besides, the His_6 tag at C terminus could not bind to the Ni NTA Resin either. So, I moved the His_6 tag to the N terminus, upstream of GST. To remove the His tag post-purification, I added a TEV enzyme cleavage site (Glu-Asn-Leu-Tyr-Phe-Gln-Gly) between the GST tag and the mtSSB protein. The cDNA of His_6-GST-mtSSB was ordered from BioBasic. It was transferred to pET28b(+) vector using the same method as described above.

The plasmid encoding either TWINKLE or mtSSB were transferred to DH5α chemically competent *E.coli* cells, and cells were grown on Luria-Bertani (LB)-agar plate with 100 μg/ml ampicillin overnight at 37 ℃. A single colony was used to inoculate LB medium (2ml) for an overnight growth at 37 ℃. The plasmid was extracted using Monarch® Plasmid Miniprep Kit and sent to GENEWIZ for sequencing. After the gene sequence was verified, the plasmid was transferred to Rosetta (DE3) chemically competent *E.coli* cells for expression. These cells were grown on an LB plate with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol (Rosetta-specific selection marker) overnight at 37 ℃. A single colony on this plate was picked up and grown in 100mL LB medium with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol overnight with rotary shaking at 37 ℃. The culture was transferred to 4×1L LB medium with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol in a 1:50 ratio and incubated at 37 ℃ with 200 rpm rotary shaking until the optical density (OD) at 600 nm reached 0.6. Flasks were cooled down in deli case to 16 ℃ and IPTG was added to a final concentration of 0.5 mM. Incubation was continued at 16 ℃ with 200 rpm rotary shaking for 20 hours. The cells were harvested by centrifugation at 5000xg.

Protein purification

Purification of His-tagged recombinant mitochondrial DNA helicase TWINKLE.

All steps of protein purification were done at 4 ℃. Frozen pellets of induced cells (harvested form 1L culture) were resuspended in 40 ml of TWINKLE lysis

buffer (Table 1) containing protease inhibitor cocktail (Sigma Aldrich, cat. #P2714). The cell suspension was sonicated using Qsonica Q125 sonicator for 20 minutes with a running/pausing time of 20s/20s. This lysate was centrifuged at 16000xg for 1 hour to remove all cell debris. The supernatant was loaded onto 5ml of HisPur® Ni NTA resin (Thermo Fisher) and incubated overnight with gentle rocking to allow Hig-tagged TWINKLE binding to the resin. The Ni resin was washed with TWINKLE Buffer A (Table 1), using rounds of resin resuspension and centrifugation, until the 280-nm UV absorbance of the buffer supernatant dropped to near-0. Then the protein bound to Ni NTA Resin was eluted by addition of 5 ml of TWINKLE Buffer B (Table 1) to the resin, resuspension, and centrifugation, repeated three times. Eluted fractions were examined for protein composition with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); the fractions were mixed with 4×Laemmli buffer (Bio-Rad) and boiled for 10 minutes; boiled samples were loaded on a 12% SDS-PAGE gel (Table 2), and the gel was run at 200V; stained with by Coomassie blue.

The fractions eluted from last step were pooled and loaded on Mono Q anion exchange column (Cytiva) with TWINKLE buffer A. This column was eluted by TWINKLE buffer C (Table 1) with a gradient from 0% to 100%. The isoelectric point (pI) of TWINKLE is 7.9, so most of this protein did not bind to the column. Therefore, the flow through was collected and loaded on a 5-mL Hitrap Heparin column (Cytiva) pre-equilibrated with TWINKLE buffer A. The column was eluted by TWINKLE buffer C (Table 1), and all the fractions were collected and examined by SDS-PAGE. The fractions that contained TWINKLE helicase were pooled and separated by HiLoad Superdex 200 16/600 column (Cytiva) on FPLC. The aggregates peak at 47 mL was discarded and the other peaks were collected. Purified TWINKLE helicase was flash frozen in liquid nitrogen and stored in -80℃ freezer until use.

Purification of His-GST-tagged recombinant mitochondrial single-stranded DNA

binding protein (mtSSB).

The frozen pellets of induced cells (harvested form 1L medium) were resuspended in 40ml of mtSSB lysis buffer (Table 3) with protease inhibitor cocktail (Sigma Aldrich, cat. #P2714). The cell suspension was sonicated using Qsonica Q125 sonicator for 20 minutes with a running/pausing time of 20s/20s. This lysate was centrifuged at 16000xg for 1 hour to remove all the cell fragments. The supernatant was filtered with 0.22μM filter to further remove any particulates. The protein solution was loaded on a 5-ml HiTrap Ni column (Cytiva) pre-equilibrated with mtSSB Buffer A and eluted by mtSSB Buffer B (Table 3). The fractions were collected and examined by SDS-PAGE.

The fractions eluted from Ni column were pooled and dialyzed against mtSSB Buffer A to remove imidazole. The recombinant protein was digested with His-tagged TEV protease (Purified in my lab) in a ratio of 20:1 at 4℃ overnight, to cleave His-GST tag. After TEV cleavage, the solution was loaded on a 5-ml HiTrap Ni column (Cytiva) equilibrated with mtSSB Buffer A (Table 3). The column was then washed with mtSSB Buffer B (Table 3). MtSSB is not His-tagged at this point, so most of this protein cannot bind to the Ni column. The flow through fractions containing untagged mtSSB were thus collected and loaded on a Mono Q column pre-washed with mtSSB Buffer A. The flow through was collected and loaded on a 5-ml HiTrap Heparin column (Cytiva) with mtSSB Buffer A. The column was eluted with mtSSB Buffer C (Table 3) and all the fractions were examined with SDS-PAGE. Glycerol was added to the purified mtSSB to a final concentration of 10%. Then this protein was flash frozen with liquid nitrogen and stored in -80℃ freezer.

The concentration for TWINKLE and mtSSB was measured by bicinchoninic acid (BCA) assay using Novagen BCA Protein Assay Kit. Protein standards were made by a serial dilution of the BSA solution provided in the kit (Table 4.). 50 μL of each standard and proteins were pipetted into test tubes and 1 ml of BCA working reagent was added. The solution was mixed by gentle vortex and incubate at 37℃ for

30 minutes. The standards and protein samples were transferred to a clean cuvette and their absorbances at 562 nm were measured with Eppendorf BioSpectrometer. The standard curve was automatically calculated by the BCA assay program on the BioSpectrometer. The protein concentration was then calculated with the standard curve. All concentrations for TWINKLE helicase in this paper are calculated based on TWINKLE being a hexamer. All concentrations for mtSSB in this paper are calculated based on mtSSB being a tetramer.

Nucleic acid substrate preparation.

The DNA sequences of the oligonucleotides used for TWINKLE and mtSSB activity tests are shown in Table 5. The oligos were ordered from Eton Bioscience, Inc. (San Diego, CA) The 5' tail strand was ^{32}P-labeled at the 5' hydroxyl using T4 Polynucleotide Kinase (T4 PNK, New England Biolabs). The reaction components are shown in Table 6. The mixture was incubated at 37℃. After 10 minutes, 1 µl of 100 µM unlabeled ATP was added to the mixture to ensure every DNA molecule is phosphorylated. After 30 minutes, the reaction was stopped by heating the reaction solution to 95 ℃ for 10 minutes, to heat-inactivate PNK.

The fork DNA was prepared by annealing of two ssDNA strands. The ssDNA was mixed as shown in Table 7. The mixture was incubated at 95℃ for 5 minutes to denature any secondary structure in ssDNA and slowly cooled down to 30℃ over a period of 20 minutes in the thermocycler.

DNA binding and unwinding assays.

TWINKLE helicase DNA unwinding activity assay

To test the DNA unwinding activity of TWINKLE helicase, 0.4 nM of TWINKLE helicase was mixed with reaction buffer containing magnesium ion and ATP required for helicase activity (Table 8). The reaction was started by adding 2 nM

of radiolabeled fork DNA and incubated at 37℃. Every 30 minutes, 2 μL of solution were pipetted out and quenched by 0.5 μL of DNA loading buffer (0.1M EDTA included to chelate magnesium). The samples were loaded on 10% Native DNA PAGE (Table 9) and run at 100V. The gel was imaged using Typhoon 2000.

MtSSB DNA binding assay

I used both radio labeled and fluorescein amidite (FAM) labeled ssDNA to test the DNA binding activity of mtSSB. For fluorescein labeled DNA method, 10 μM of mtSSB was mixed with reaction buffer. (Table 10.). The reaction was started by adding 1 μM of FAM labeled ssDNA and incubated at 37℃. The reaction was quenched by DNA loading buffer (EDTA included). The samples were loaded on 10% native DNA PAGE and run at 100V. The gel was imaged by gel Gel Doc EZ imager (Bio-Rad).

For ^{32}P-labeled DNA assay, 10 nM of mtSSB was mixed with reaction buffer. (Table 10.). The reaction was started by adding 1 nM of radio labeled ssDNA and incubated at 37℃. The reaction was quenched by DNA loading buffer (EDTA included). The samples were loaded on 10% native DNA PAGE and run at 100V. The gel was imaged by Typhoon 2000.

DNA unwinding assay in the presence of mtSSB with native PAGE analysis.

0.4 nM of TWINKLE helicase and 10 nM of mtSSB were mixed with reaction buffer. (Table 11) The reaction was started by adding 1nM of ^{32}P-labeled fork DNA and incubated at 37℃. Every 30 minutes, 2 μL of solution was pipetted out and quenched by DNA loading buffer (EDTA included). The samples were loaded on 10% native DNA PAGE and run at 100V. The gel was imaged by Typhoon 2000 and quantified with Image J.

DNA unwinding assay in the presence of mtSSB with size-exclusion

chromatography analysis.

10 μM of TWINKLE helicase and 50 μM of mtSSB were mixed with reaction buffer. (Table 12.) The reaction was started by adding 10 μM of unlabeled fork DNA and incubate at 37℃. After 2 hours, the reaction was quenched by 100 μM EDTA. The samples were loaded on FPLC system and separated by Superose 6 10/300 column. The fractions were collected and examined with SDS-PAGE.

Table 1: TWINKLE helicase purification buffers

Component	Lysis Buffer	Buffer A	Buffer B	Buffer C
Tris-HCl	50 mM	30 mM	30 mM	30 mM
Glycerol	10%	20%	20%	20%
Tween 20	0.1%	0.1%	0.1%	0.1%
$MgCl_2$	5 mM	5 mM	5 mM	5 mM
NaCl	500 mM	310 mM	310 mM	1 M
DTT	0.2 mM	0.2 mM	0.2 mM	0.2 mM
Imidazole	0	0	300 mM	0
pH	7.5	7.5	7.5	7.5

Table 2: SDS-PAGE recipe

Component	12.5% Stacking Gel	12.5% Resolving Gel
40% Acrylamide	255 µl	1.56 ml
H_2O	1.45 ml	2.09 ml
1.5 M Tris (pH 8.8)	250 µl	0
1 M Tris (pH 6.8)	0	1.25 ml
10% w/v SDS	20 µl	50 µl
10% w/v APS	20 µl	50 µl
TEMED	2 µl	5 µl

Table 3: mtSSB purification buffers

Component	Lysis Buffer	Buffer A	Buffer B	Buffer C
NaCl	137 mM	137 mM	137 mM	1 M
KCl	2.7 mM	2.7 mM	2.7 mM	2.7 mM
Na_2HPO_4	10 mM	10 mM	10 mM	10 mM
KH_2PO_4	1.8 mM	1.8 mM	1.8 mM	1.8 mM
DTT	0.5 mM	0.5 mM	0.5 mM	0.5 mM
Triton X-100	1%	0	0	0
Imidazole	0	0	300 mM	0
pH	7.4	7.4	7.4	7.4

Table 4: BCA reaction standards

Sample	BSA Concentration/(μg/ml)
A	2000
B	1500
C	1000
D	750
E	500
F	250
G	125
H	25

Table 5: Oligonucleotides for DNA unwinding experiment

Name	Sequence
FAM labeled 5' tail strand	5'- /FAM/ TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTC TAA TTA ATA TAA TTA TAA TAA TAT ATA ATA ATT AAT ATG GGG -3'
3' tail strand	5'-CAT ATT AAT TAT TAT ATA TTA TTA TAA TTA TAT TAA TTA GTT TTT TTT TTT TTT T -3'
5' tail strand	5'- TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTC TAA TTA ATA TAA TTA TAA TAA TAT ATA ATA ATT AAT ATG GGG -3'

Table 6: Polynucleotide kinase (PNK) reaction recipe

Component	Volume/μl	Final concentration
1 mM 5' tail strand DNA	1	100 nM
PNK Buffer	1	/
T4 PNK	0.5	/
[γ-^{32}P] ATP	1.5	/
H_2O	5	/
100 μM ATP (add after 10mins)	1	10 μM
Final volume	10	/

Table 7: Fork DNA assembly recipe

Component	Volume/μl	Final concentration
100 nM 5' tail strand	4	20 nM
100 nM 3' tail strand	5	25 nM
H_2O	11	/
Final volumn	20	/

Table 8: TWINKLE activity test mixture components

Component	P:D=1:1 group	P:D=1:5 group	Blank group
Fork DNA	1 nM	1 nM	1 nM
TWINKLE	1 nM	0.2 nM	0
HEPES	20 mM	20 mM	20 mM
$MgCl_2$	5 mM	5 mM	5 mM
ATP	4.5 mM	4.5 mM	4.5 mM
DTT	5 mM	5 mM	5 mM
BSA	50 μg/ml	50 μg/ml	50 μg/ml
Glycerol	7%	7%	7%
pH	7.5	7.5	7.5

Table 9: 10% DNA native PAGE recipe

Component	Volume
40% acrylamide	3.75 ml
10×TBE buffer	1.5 ml
10% APS	80 μl
H_2O	9.75 ml
TEMED	8 μl

Table 10: mtSSB activity test mixture components

Component	FAM group	Radio labeled group
Labeled 5' tail ss DNA	1 µM	1 nM
mtSSB	10-40 mM	40 nM
HEPES	20 mM	20 mM
$MgCl_2$	5 mM	5 mM
ATP	4.5 mM	4.5 mM
DTT	5 mM	5 mM
BSA	50 µg/ml	50 µg/ml
Glycerol	7%	7%
pH	7.5	7.5

Table 11: Radio labeled fork DNA unwinding assay components

Component	TWINKLE+mtSSB group	TWINKLE group
TWINKLE	0.2 nM	0.2 nM
mtSSB	10 nM	0
HEPES	20 mM	20 mM
$MgCl_2$	5 mM	5 mM
ATP	4.5 mM	4.5 mM
DTT	5 mM	5 mM
BSA	50 µg/ml	50 µg/ml
Glycerol	7%	7%
pH	7.5	7.5

Table 12: Unlabeled fork DNA unwinding assay components

Component	TWINKLE+mtSSB group	TWINKLE group
Fork DNA	1 mM	1 mM
TWINKLE	1 mM	0.2 mM
mtSSB	10 mM	0
HEPES	20 mM	20 mM
$MgCl_2$	5 mM	5 mM
ATP	4.5 mM	4.5 mM
DTT	5 mM	5 mM
BSA	50 µg/ml	50 µg/ml
Glycerol	7%	7%
pH	7.5	7.5

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