Mechanisms of Gene Expression – Team Project 4

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Title

Mechanisms of DNA Polymerase III under replication stress.

Abstract

DNA replication requires coordinated action between DNA polymerase (DNAP) and helicase to ensure efficient unwinding and synthesis (Lo and Gao, 2022). Previous work has shown that when under stress, T7 DNAP can lead to fork inactivation and locking (Jia *et al.*, 2024). However, whether these mechanisms occur in *Escherichia coli* remains unknown. Here, we propose to conduct the in-depth characterization of the interaction between DNA polymerase III (PolIII) and the replication fork in *E. coli* to determine whether similar fork-locking dynamics occur. We aim to identify the role of exonuclease activity in maintaining fork integrity. Understanding these mechanisms in *E. coli* will provide new insights into replication dynamics and fork stability in bacterial systems.

Key-words

DNA replication, DNA polymerase III, exonuclease, replication fork locking, *E. coli*

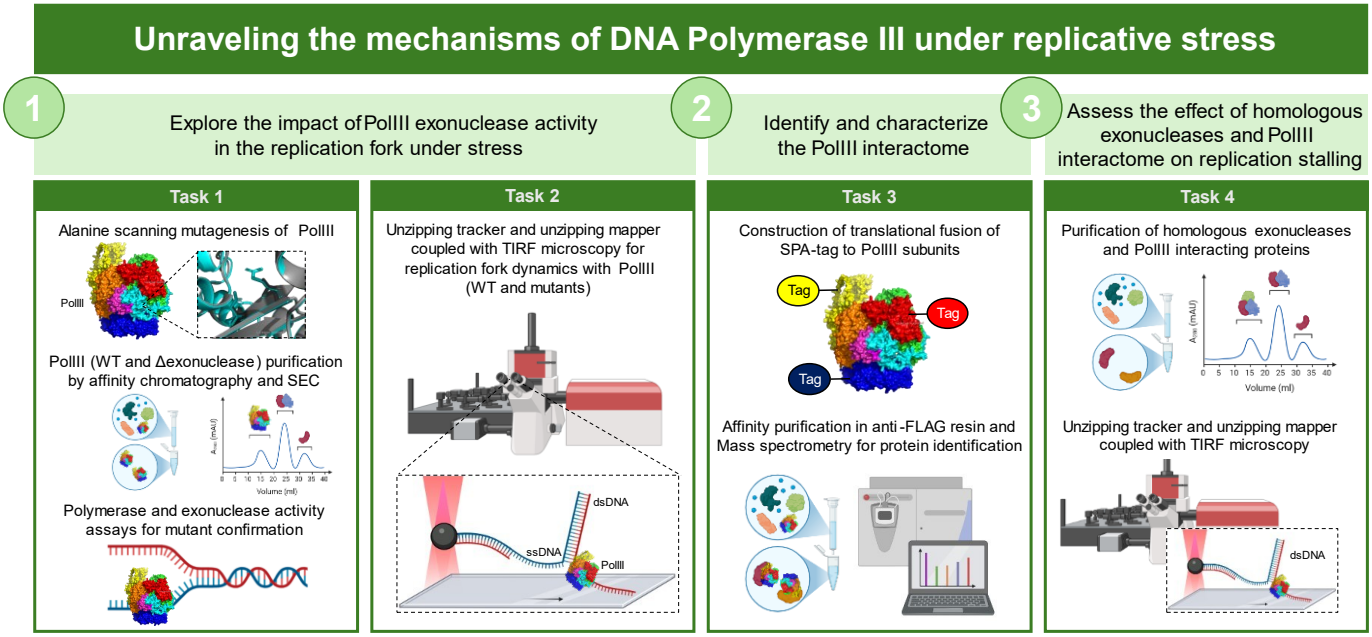

Image created using *BioRender*, *Alphafold3* structural models and adaptions from Ian *et al*, 2024

State of the Art

DNA replication is a tightly regulated process essential for genome stability. One major challenge associated with its regulation is the advent of replication stress, which may occur when the coordination between the helicase and polymerase is disrupted. It has been shown that, in bacteriophage T7, this often leads to helicase lagging behind or dissociating entirely, leaving the polymerase to struggle against DNA reannealing—ultimately stalling replication and threatening genome integrity (Pandey and Patel, 2014; Jia *et al.*, 2025).

While T7 serves as a simple model for studying replication dynamics, expanding this research to more complex systems like *Escherichia coli* could offer deeper insights. Bacterial replication involves additional regulatory mechanisms, and comparing different organisms may reveal conserved and unique strategies for managing replication stress (Reyes-Lamothe, Sherratt and Leake, 2010).

E. coli has a more complex replication mechanism that involves five DNA polymerases with distinct roles. DNA polymerase I (PolI) processes Okazaki fragments and PolII assists in proofreading, while PolIV and PolV are responsible for repairing DNA damage associated with stress conditions. *E. coli* PolIII is the main replicative enzyme, and it's organized into a core (α, ε and θ subunits), a sliding clamp (the β subunit) and a clamp loader (τ and γ subunits). In the core, the α subunit assures polymerase activity while the ε subunit provides 3'→5' exonuclease proofreading function (Fijalkowaska, Schaaper & Jonczyk, 2012).

In *E. coli*, the intrinsic exonuclease activity of PolIII likely plays a key role in resecting nascent DNA and resetting stalled replication forks. Identifying the critical residues involved in this process could clarify how exonuclease activity may help helicase re-engage and PolIII resume synthesis (Canal *et al.*, 2024). Additionally, introducing exonucleases from other species, such as the homolog Trex2, may further aid replication restart, providing new strategies to counteract replication stress and improve our understanding of helicase-polymerase coordination. Trex2 is a small human exonuclease, homologous to PolIII, responsible for repairing damaged replication forks (Hasty, 2021). Additionally, its disruption has showed to increase mouse susceptibility to skin cancer, which highlights the importance of investigating the mechanism of action of these type of enzymes (Parra *et al*, 2009).

Lastly, exploring other proteins that interact with the PolIII in *E. coli* could provide valuable insights into how replication may be restarted. Many polymerase-binding

proteins play crucial roles in DNA replication and, as such, may also aid in fork stalling and promoting replication restart. Identifying these proteins that interact with the replication machinery could enhance our understanding of not only *E. coli* replication control but also the broader mechanisms underlying replication restart across different organisms (Kasho and Katayama, 2013; Fang, Engen and Beuning, 2011).

Objectives

The overarching objective is to unravel the mode of action of DNA polymerase III under replication stress. The specific goals are defined as follows:

Aim 1 (Task 1 and Task2): Identify key residues for PolIII exonuclease activity and investigate its impact on the replication fork under stress.

Aim 2 (Task 3): Apply a global approach to identify proteins that interact with PolIII.

Aim 3 (Task 4): Assess the effect of increasing concentrations of PolIII-binding proteins and homologous exonucleases on fork processing.

Detailed description

Considering our goals, we outlined these tasks:

Task 1: Mutational analysis of DNA polymerase III. We will use the *AlphaFold3* model of the ε subunit of PolIII, which is known to possess exonuclease activity, along with the identification of residues conserved across PolIII homologues to guide alanine scanning mutagenesis studies. Wild-type and PolIII variants will be produced as His-tagged proteins in *E. coli* and purified using a Ni^{2+}-column followed by size exclusion chromatography (SEC) (Lin, Bin and Zhang, 2011). Activity assays will then be performed with purified proteins to assess the impact of the mutations on enzymatic function (polymerase and exonuclease).

Task 2: Single-molecule analysis of replication fork dynamics. To evaluate the impact of PolIII exonuclease activity on the replication fork under stress, we will employ single-molecule tracking approaches, including unzipping tracker and mapper assays, which will allow us to monitor replication fork progression, stalling, and degradation in real time. Replication fork substrates will be designed with fluorescent markers to facilitate the visualization of fork movement and processing events (Jia *et al.*, 2024). Wild-type and mutant PolIII variants will be introduced to assess their impact on fork stability. Experiments will be conducted in a microfluidic chamber to capture the effects of PolIII and exonuclease activity and will be analyzed using TIRF microscopy. Fork stability and

degradation will be quantified under normal and stress conditions, such as nucleotide depletion and DNA damage, to determine the effect of PolIII exonuclease activity in these conditions.

Task 3: Identification of PolIII interactome by affinity purification followed by mass spectrometry (AP-MS) (Morcinek-Orłowska *et al.*, 2021). We will insert a translational fusion of SPA-tag to different subunits of PolIII (encoded in different genes, hereinafter called *polIII* for simplicity) in an E. *coli* replicative plasmid. The plasmid containing the fusion will be transferred to the *polIII* deletion mutant, so that the SPA-tagged version will be the only copy present in the cell. We will then perform affinity chromatography using an anti-FLAG resin, followed by mass spectrometry to identify interacting proteins.

Task 4: Impact of PolIII-binding proteins and homologous exonucleases on fork processing. To assess how PolIII-binding proteins and homologous exonucleases influence replication fork stability, we will perform *in vitro* unzipping tracker and mapper assays. PolIII-binding proteins and homologous exonucleases, such as Trex2, will be purified using affinity purification followed by SEC (as described in Task 1). The effect of increasing concentrations of these proteins on replication fork stability will be evaluated using unzipping tracker and mapper assays, allowing real-time visualization of interactions between PolIII, its binding partners, and exonucleases at the replication fork (see Task 2).

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