Structural basis for functional cooperation between proteins involved in the processing of RNA primers during mitochondrial DNA replication

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Human mitochondrial DNA (mtDNA) is a circular, double-stranded molecule of 16.6 kilobase pairs whose replication and maintenance are essential for cellular function, as mutations in mtDNA cause severe mitochondrial diseases. mtDNA replication differs fundamentally from nuclear replication and relies on a dedicated set of mitochondrial proteins. In the widely cited strand displacement model, leading strand synthesis initiates at the heavy-strand origin. When approximately two-thirds of the genome have been replicated, the light-strand origin becomes exposed and adopts a stem-loop structure. After replication is completed, RNA primers at the 5′ ends of nascent strands must be removed. Defects in this step lead to the formation of pathogenic mtDNA species and contribute to diverse mitochondrial disorders. Although recent studies have identified functional interactions among human mitochondrial ribonuclease H1 (RNase H1), the mitochondrial 5′-exonuclease G (EXOG), and DNA polymerase gamma (Pol γ), the precise mechanism of RNA primer removal in human mitochondria remains only partially understood. To address this gap, I investigated two distinct RNase H1-centred interactions: its cooperation with EXOG in terminal RNA primer processing and its functional interplay with Pol γ during mtDNA replication. The work integrates biochemical and kinetic assays, including surface plasmon resonance (SPR) and biolayer interferometry (BLI), mutational analysis, structural modelling using AlphaFold, X ray crystallography, and cryo electron microscopy (cryo-EM).

In the first part of this project, I examined the functional cooperation between RNase H1 and EXOG. SPR analysis mapped the primary interaction surface to the catalytic domain of RNase H1. Biochemical reconstitution demonstrated that both enzymes are required together to achieve complete excision of RNA primers *in vitro*, a result that neither protein can achieve on its own. Furthermore, EXOG partially restored the impaired activity of a disease associated RNase H1 variant, suggesting potential clinical relevance. To investigate the structural basis of this cooperation, I applied an integrated approach combining crystallisation attempts with AlphaFold2 Multimer predictions. While crystallography did not yield a complex structure, computational modelling identified key interfacial residues that were subsequently validated through site directed mutagenesis and biochemical activity assays.

The second part of the thesis explored the interplay between RNase H1 and Pol γ. SPR experiments revealed a direct interaction mediated by the catalytic domain of RNase H1. This association enabled a reciprocal functional relationship. DNA synthesis by Pol γ stimulated RNase H1 to achieve complete primer removal, whereas a catalytically inactive RNase H1 variant halted Pol γ during gap-filling synthesis, acting as a physical obstacle. Although biochemical data supported the existence of a ternary assembly, cryo-EM analysis revealed substantial conformational heterogeneity that prevented high resolution visualisation of RNase H1. To address this limitation, AlphaFold3 modelling generated a plausible structural model consistent with the biochemical observations and suggested a spatial arrangement that could facilitate coordinated primer removal during mtDNA replication termination.

Together, these findings reveal complementary mechanisms through which RNase H1 collaborates with distinct enzymatic partners to ensure complete RNA primer removal and proper replication termination in human mitochondria. This work also defines the specific biochemical conditions under which RNase H1 is capable of complete RNA primer cleavage *in vitro*, clarifying previously unresolved observations. By integrating structural, biochemical, and kinetic evidence, the thesis expands current understanding of mitochondrial RNA primer maturation and highlights the contribution of RNase H1 activity to replication termination and mitochondrial genome stability.