The Institute of Cancer Research

PHD STUDENTSHIP PROJECT PROPOSAL

PROJECT DETAILS	
Project Title	Unravelling the mechanism of DNA-induced replication stalling
Short Project Title	**DNA-induced replication stalling**

SUPERVISORY TEAM	
Primary Supervisor	Dr. Gideon Coster
Associate Supervisor(s)	Prof. Jessica Downs
Secondary Supervisor	Prof. Jonathon Pines

DIVISIONAL AFFILIATION	
Primary Division	Cancer Biology
Primary Team	Genome Replication
Site	Chelsea

PROJECT PROPOSAL

Why study replication?

Cancer is a disease of uncontrolled proliferation. As cells are pushed into shorter cell cycles, they experience replication stress, which drives genomic instability and disease progression [1]. This is especially true for colorectal cancer, the third most prevalent cancer in the world - 15% of cases are caused by inactivation of mismatch repair (MMR), the major repair pathway of replicative errors. This means that mistakes during normal DNA replication are sufficient to drive tumorigenesis.

Why study repeat replication?

Replication accuracy is not equal across the genome. Certain regions, especially repetitive and structure-prone sequences, present a challenge to the replication machinery. In the past three decades, instability of short repeats (microsatellite instability) has been widely used as a molecular diagnostic and prognostic cancer marker [2]. However, recent work suggests that these unstable sequences may also play a direct causative role [3, 4].

It is generally accepted that repetitive DNA sequences are unstable because they can form unusual DNA secondary structures. However, whether DNA on its own can drive replication stalling has been unclear.

Recent unpublished data from our lab reveal that certain repetitive sequences can indeed lead to replication stalling in vitro. This is the first direct evidence that DNA can interfere with the eukaryotic replisome. These findings raise many exciting questions regarding the mechanism of replication stalling and how the replisome ultimately replicates past these challenging sequences. Key open questions are:

>> What are the molecular events that underlie DNA-induced replication stalling?

>> What are the factors and mechanisms that facilitate accurate replication of repeats?

Using a powerful combination of in vitro biochemistry, proteomics, structural biology and genetics, this project is aimed at defining the dynamics of replication within repetitive sequences and discovering novel factors required for their faithful duplication.

PROJECT AIMS

The overall goal of this project is to determine the dynamics of repeat replication using a highly defined in vitro replication system. By studying this process in isolation, we will gain direct insight into why repeats are a block to replication and how they are resolved and replicated accurately.

The PhD candidate will carry out the following specific tasks:

>> Define the molecular mechanism of **replication stalling** at repetitive sequences

>> Determine the nature of replication-induced **unusual DNA structures**

>> Collaborate to visualize DNA secondary structures by **Atomic Force Microscopy** (AFM)

>> Employ proteomics of replication competent S-phase extracts to **identify novel factors**

>> Confirm and further **explore top novel factors** using biochemistry, genetics and cell biology

RESEARCH PROPOSAL

General approach and preliminary results

DNA replication requires the coordinated activity of a multi-protein machine termed the replisome, which combines the action of enzymes such as a replicative helicase and polymerases. This remarkably complex reaction has been recently reconstituted in vitro using purified budding yeast proteins [5]. This exciting breakthrough allows us for the first time to directly study eukaryotic DNA replication in unprecedented molecular detail.

Evidence from genetic studies suggest that certain sequences interfere with replication [6-9]. However, it remains unclear whether stalling is directly caused by DNA itself or mediated by other factors such as DNA-bound proteins. Studies in vitro have thus far been confined to analysis of isolated polymerases using primer extension assays. Such studies show that short ssDNA substrates that have the potential to form secondary structures can inhibit DNA synthesis. However, whether such structures can actually form in the context of the complete replisome is unknown.

To address these key questions we have utilised the in vitro replication system and tested a large range of repetitive sequences. **Our unpublished data indicate that several repeat sequences can indeed stall replication**. These results open up many important and exciting avenues of research. What is the mechanism of stalling? Is the helicase stalled or is it the polymerase? What allows the replisome to deal with these impediments?

This project will build on these initial observations and will expand on them through three broad aims:

1. Formation and nature of replication-dependent DNA structures

Because secondary structures are thought to cause repeat instability, it is important to establish whether replication can induce them. While studies in vivo are indeed suggestive of this [6], whether replication alone is sufficient for structure formation is unclear. We also know little about the role of local DNA context, DNA topology and strand specificity in replication-induced structure formation and stability.

1.1. Secondary structures - requirements and stability

The PhD candidate will employ a variety of DNA substrates that contain a specific replication origin and repetitive and structure-prone sequences, such as microsatellites, trinucleotide repeats, inverted repeats and G4-forming sequences. These substrates will be used in replication reactions in vitro and probed for DNA secondary structures using a wide selection of structure-specific nucleases in conjunction with native gel electrophoresis. If structures are sufficiently frequent and stable, the PhD candidate will explore which replisome components are required for structure formation. We will define how local DNA context affects structure formation and stability. If structures are rare or transient, we will test whether they can be induced or stabilized by a replication competent S-phase extract. If so, we will identify novel responsible factors by proteomic approaches.

In addition, the PhD candidate will benefit from our ongoing collaboration with the lab of Dr. Alice Pyne, University of Sheffield (https://pyne-lab.uk/). The Pyne lab are pioneers in the use of cutting-edge high resolution Atomic Force Microscopy (AFM) to visualize single DNA molecules. This approach will allow us to directly visualize DNA secondary structures, with the aim of detecting replication-induced structures.

1.2. DNA topology

Unwinding of DNA generates torsional strain which must be relieved by topoisomerases to allow replication to proceed. In addition, the formation of DNA secondary structures is often affected, or even requires, negative supercoiling. We will therefore test whether topology has any role in structure formation. First, we will compare structure formation using circular versus linear templates. We will also employ different types and levels of topoisomerases. Finally, we will also pre-treat plasmid substrates with topoisomerases to test the effects of their initial topological state, and use nicked plasmids as controls. Together, these experiments will define the role topology plays in structure formation before and during replication.

2. Effects of repeats & DNA secondary structures on replication dynamics

What are the effects of repeats on DNA replication? Are these effects indeed due to formation of DNA secondary structures? In addition to self-induced structures, the replication machinery must also contend with pre-existing structures introduced by other DNA metabolic pathways. This part of the proposal is aimed at defining why only certain sequences stall the replisome, how they do so and whether stalling is mediated by DNA secondary structures.

2.1. Global replication dynamics

Our recent findings reveal that the replisome stalls at certain repeats. We will ask whether replication initiation, DNA unwinding, leading and lagging strand synthesis, and replisome stability are affected by repeats and by secondary structures. In addition to a range of substrates already available in the lab, the PhD candidate will construct vectors that harbour a single replication origin and a site-specific pre-formed DNA structure using an oligo-replacement strategy. Structures will be positioned on either the leading or lagging strand template. We will monitor replication and the composition of the associated replisome. If the replisome is intact, we will employ single particle electron microscopy to elucidate its configuration and establish whether it is stalled by structured DNA. If it is unstable, we will search for factors required to maintain its stability on DNA using proteomics of S-phase extracts. We will also test whether Okazaki fragment maturation is affected. Finally, if we find that the helicase is affected, we will perform DNA unwinding assays with purified CMG (the eukaryotic replicative helicase) and define which structures (if any) impair its activity. If instead we find that the helicase is unaffected, this will point to the polymerase as being affected. We will therefore test the ability of a hyperactive polymerase epsilon variant [10] to rescue stalling.

2.2. Origin distance and orientation

It has been observed in several organisms that the orientation of a replication origin relative to repeat sequences can have a significant effect on repeat instability [6-9]. This effect seems stronger when repeats are in close proximity to an origin. To understand the nature of this phenomenon, we will construct a series of substrates with different spacing and orientation of the origin relative to repeats and study their replication dynamics in vitro. We will also manipulate Okazaki fragment size and processing. Finally, we will explore the role of the ssDNA binding protein RPA, and whether limiting amounts of RPA exacerbate structure formation.

3. Factors that enable faithful replication of structure-prone DNA

What mechanisms ensure that repeats are replicated accurately? Are different classes of repeats and structures resolved by distinct mechanisms? This part of the proposal is aimed at identification of novel factors that may assist the replisome in replicating challenging templates.

3.1. Proteomic and candidate approach for identification of novel factors

The key advantage of the in vitro system is that it is highly defined. However, it may lack key factors to enable efficient and/or accurate replication of repeat sequences. We will assay replication fidelity by performing next generation sequencing on in vitro replicated templates. We will then test whether a replication-competent S-phase extract supports more accurate or efficient repeat replication relative to a minimal or complete purified replisome [11]. If so, we will combine candidate and proteomic approaches to discover novel factors. If not, we will identify which replisome components perform these functions and how.

Altogether, this project will provide novel insight into the formation and resolution of replication-induced toxic DNA structures, establish how repeats affect the replisome and reveal how the replisome ultimately deals with these challenges to support accurate genomic replication. The results may help identify novel cancer targets and may improve our use of microsatellite instability as a diagnostic and prognostic cancer marker.

LITERATURE REFERENCES

1. Hills, S.A. and J.F. Diffley, "*DNA replication and oncogene-induced replicative stress".* **Current Biology**, 2014. **24**(10): p. R435-44.
2. Umar, A., et al., "*Revised Bethesda Guidelines for Hereditary Nonpolyposis Colorectal Cancer (Lynch Syndrome) and Microsatellite Instability".* **Journal of the National Cancer Institute**, 2004. **96**(4): p. 261-268.
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11. Kurat, C.F., et al., "*Chromatin Controls DNA Replication Origin Selection, Lagging-Strand Synthesis, and Replication Fork Rates".* **Molecular Cell**, 2016. **65**(1): p. 117-130.

CANDIDATE PROFILE

Note: the ICR's standard minimum entry requirement is a relevant undergraduate Honours degree (First or 2:1)

Pre-requisite qualifications of applicants	B.Sc. or M.Sc. (First or 2:1, or equivalent) in any of the following disciplines: >> Life Sciences / Biology >> Biochemistry >> Biomedical Sciences >> Molecular Biology >> Genetics
Intended learning outcomes	During the course of this studentship, the PhD candidate will be fully supported and encouraged to develop multiple skills and become proficient in the following: >> Molecular biology techniques (cloning, PCR, western blotting etc…) >> Protein expression and purification using a variety of systems (bacteria / yeast) >> Experimental design and interpretation >> Biochemical analysis of complex reactions >> Reading and critical analysis of published journal articles >> Writing and publishing original research papers >> Presentation of scientific projects to an expert audience
ADVERTISING DETAILS	
Project suitable for a student with a background in	☒ Biological Sciences ☐ Physics or Engineering ☒ Chemistry ☐ Maths, Statistics or Epidemiology ☐ Computer Science ☐ Other (provide details)
Keywords	**1. DNA Replication**
	2. Genome stability
	3. Biochemistry
	4. In vitro reconstitution
	5. Repeat instability
	6. Genetics