The Institute of Cancer Research

PHD STUDENTSHIP PROJECT PROPOSAL

FUNDER DETAILS	
Studentship funded by:	Medical Research Council - Doctoral Training Partnership (MRC DTP)
Funder specific requirements:	All MRC DTP students will attend taught courses one day a week for the first nine months of the PhD. This training will cover computational and thematic science training as well as core and transferable skills. Students will spend the remainder of the four years on their PhD project full time with monthly cohort activities.
PROJECT DETAILS	
Project Title:	**Composition and function of telomeric multi-protein complexes and their regulation by ADP-ribosylation**
Short Project Title:	**Regulation of telomere homeostasis by ADP-ribosylation**
SUPERVISORY TEAM	
Primary Supervisor(s):	Dr Sebastian Guettler
Associate Supervisor(s):	Dr Oviya Inian
Secondary Supervisor:	Professor Vlad Pena
DIVISIONAL AFFILIATION	
Primary Division:	Structural Biology
Primary Team:	Structural Biology of Cell Signalling
Other Division (if applicable):	Cancer Biology

SHORT ABSTRACT

Telomere maintenance is vital to genome stability. The Shelterin complex, composed of six different proteins, protects telomeres from an illegitimate DNA damage response and orchestrates telomere extension by telomerase. The precise molecular mechanisms of telomere protection and length homeostasis are incompletely understood. How does the cell navigate the dilemma that chromosome termini are sequestered from the DNA-damage sensing

machinery to avoid lethal chromosome fusions whilst needing to be exposed to telomerase for telomere extension? In human cells, the ADP-ribosyltransferase tankyrase modifies Shelterin to enable telomerase-dependent telomere extension, presumably by modulating Shelterin stoichiometry. In this project, we aim to combine biochemical, biophysical, single-molecule fluorescence and cell-based studies to understand the relevance and regulation of Shelterin complex stoichiometry in telomere protection and length homeostasis.

BACKGROUND TO THE PROJECT

Repetitive DNA sequences at chromosome termini, the telomeres, enable chromosome end maintenance through the action of telomerase in S-phase. By extending telomeres, telomerase solves the so-called "end replication problem"[1]. Moreover, telomeric structures formed by telomere-binding proteins protect chromosome termini, which resemble DNA double strand breaks, from an illicit DNA damage response. This occurs primarily through sequestration of the DNA ends and addresses the so-called "end protection problem"[1]. The multi-protein complex decorating telomeres is known as Shelterin and is vital to both end replication and protection[1,2]. Remarkably, telomere extension requires the liberation of the DNA terminus as a telomerase substrate without triggering a DNA damage response. We aim to understand the molecular mechanisms behind this molecular tightrope walk achieved by the Shelterin complex.

Shelterin consists of six proteins: TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 (Figure 1)[1]. TRF1, TRF2 and RAP1 are present in two copies each whilst the other components occur as single copies[3]. In humans and many other species except numerous rodents, the poly-ADP-ribosyltransferase tankyrase provides an important regulatory layer to telomere homeostasis: binding to Shelterin, it ADP-ribosylates TRF1, which is thought to evict TRF1 from the complex, thereby enabling telomerase to act on telomeres[4,5].

In this project, we aim to identify and characterise different compositional states of Shelterin, their regulation by tankyrase and their physiological relevance to telomere protection and length homeostasis. As telomerase activation is a hallmark of 85-90% of all cancers, and as telomeres are dysregulated in a range of diseases[6], understanding telomere maintenance mechanisms has the potential to reveal novel vulnerabilities of telomerase-dependent cancers and deliver therapeutically valuable insights into a range of telomere spectrum disorders.

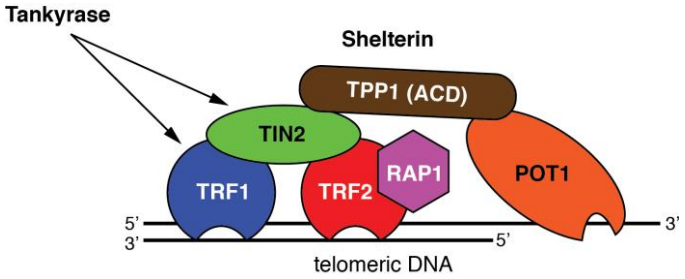

Figure 1: The telomeric Shelterin complex.
Schematic representation of the human Shelterin complex, many units of which decorate telomeric repeats.

PROJECT AIMS

- Elucidate the hierarchy of Shelterin complex assembly and its stoichiometries
- Study the impact of ADP-ribosylation on Shelterin complex stoichiometry
- Interrogate the functional impact of Shelterin stoichiometry and ADP-ribosylation on telomerase activity *in vitro*

- Interrogate the functional impact of Shelterin stoichiometry and ADP-ribosylation on telomere extension in human cells

RESEARCH PROPOSAL

It remains unknown whether the stoichiometric heterogeneity of Shelterin, detected *in vitro*, reflects different physiologically relevant states of the complex and how it impinges on telomere protection and length homeostasis. Telomere extension in human cells is thought to occur upon TRF1 eviction from Shelterin through tankyrase-dependent ADP-ribosylation[4,5]. In this project, we aim to take a biochemical, biophysical and cell-based approach to explore how cells ensure telomere protection is maintained whilst simultaneously making the telomere available to telomerase for extension.

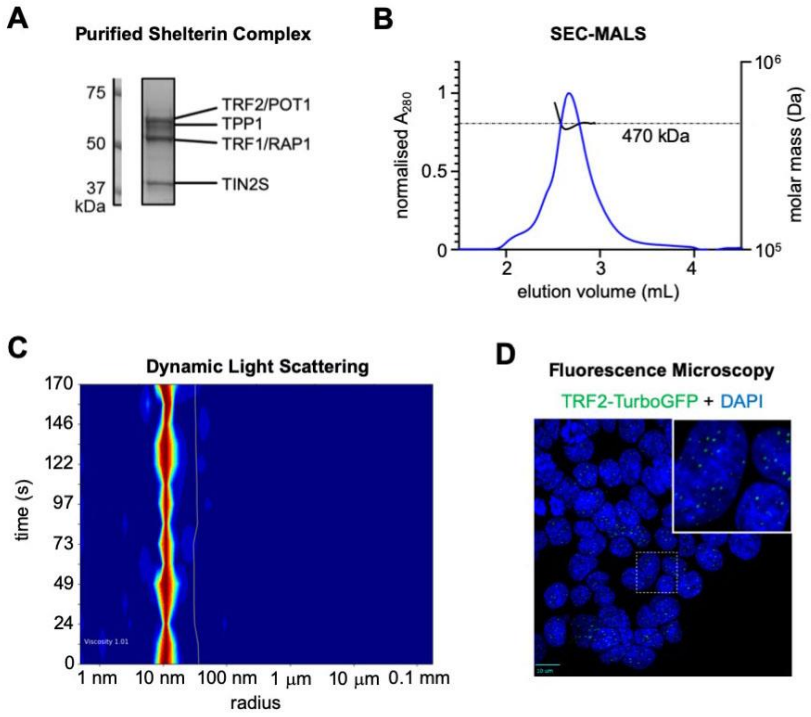

Figure 2: Biochemical reconstitution and biophysical characterisation of the telomeric Shelterin complex and its analysis in human cells.
(A) Purified Shelterin complex analysed by SDS-PAGE and Coomassie staining. **(B)** Analysis of Shelterin by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS). **(C)** Analysis of Shelterin by dynamic light scattering (DLS). **(D)** Fluorescence micrograph of HAP1 cells expressing endogenously TurboGFP-tagged TRF2.

Establishing fluorescence-based studies of the Shelterin complex

We have recently established the specific, single-site fluorescent labelling of purified recombinant proteins via a ybbR peptide tag[7,8]. To facilitate the investigation of Shelterin stoichiometries and assembly, the candidate will similarly tag different Shelterin subunits, purify the individual components and subsequently modify the tags with spectrally separable Alexa Fluor variants through maleimide coupling[7]. Following their purification, the fluorescently labelled variants will be used to assemble Shelterin complexes in a step-wise fashion on telomeric DNA. In the first instance,

we will perform electrophoretic mobility shift assays (EMSAs) to monitor assembly and discern the composition of the complexes. This will be paralleled by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS), dynamic light scattering (DLS) and mass photometry studies to determine the sizes and molecular weights of the resulting assemblies (Figure 2A-C). Once the functionality of the tagged proteins is confirmed, we will reconstitute Shelterin complexes on extended double-stranded or hybrid single/double-stranded telomeric DNA, investigating the complexes and their regulation by single-molecule fluorescence techniques, which we aim to establish in the course of this project.

Regulation of Shelterin stoichiometry and telomere extension by tankyrase

We will use purified tankyrase to explore whether and how ADP-ribosylation changes the stoichiometry of Shelterin. We will use (1) pulldown assays, (2) EMSA, (3) SwitchSense DNA binding assays, (4) SEC-MALS and mass photometry, and (5) single-molecule fluorescence approaches to qualitatively and quantitatively assess Shelterin assembly in response to tankyrase. We will provide tankyrase and its co-substrate NAD^+ either to the full complex or assess the incorporation of pre-ADP-ribosylated TRF1 to a subcomplex lacking TRF1. We will monitor the levels of TRF1 and TRF2 within the complex, relative to each other and relative to the core component TIN2 (Figure 1). In pulldown assays and potentially EMSAs, immunoblotting for ADP-ribose will enable us to track whether the ADP-ribosylated population of TRF1 or any other potentially modified Shelterin components are liberated from or retained within the complex. Tankyrase mutant variants that are either catalytically inactive or deficient in TRF1 binding will serve as controls, as will TRF1 mutants unable to bind to tankyrase.

The candidate will further explore the effect of TRF1 and tankyrase on the processivity of the telomerase reaction. Direct telomerase assays have shown that the core Shelterin complex lacking TRF1 facilitates telomerase processivity[3]. The inclusion of TRF1 and tankyrase into these assays will provide insights into the regulation of telomere extension by these factors *in vitro*.

Monitoring Shelterin composition in cells

We will next complement our biochemical and biophysical studies with cell-based experiments to test hypotheses arising from the experiments outlined above. Telomeres manifest as punctate nuclear structures in light microscopy (Figure 2D). We will use HAP1 cells with GFP-tagged TRF2 (Figure 2D) to introduce additional spectrally distinct tags on TRF1 (e.g., EBFP) and TIN2 (e.g., mPlum), using the CRISPaint technology[10]. The haploid karyotype of HAP1 cells is particularly conducive to gene editing. We will monitor the relative intensity of signals of Shelterin components by fluorescence microscopy (1) throughout the cell cycle, (2) upon tankyrase catalytic inhibition, (3) upon genetic silencing of tankyrase or pharmacological inactivation of tankyrase, or (4) upon overexpression of different tankyrase variants. Paired with quantitative telomere fluorescence *in situ* hybridisation (FISH), these microscopy studies will simultaneously provide information on Shelterin composition and telomere length, and how both respond to tankyrase activity. We will further perform cell passaging studies with telomere restriction fragment analyses to monitor telomere extension[5]. Should our biochemical studies confirm the exclusion of TRF1 by tankyrase-dependent PARylation, we will also generate HAP1 cells lacking TRF1 and assess their telomere maintenance. We will monitor telomere protection in these cells by co-staining telomeres with DNA damage markers such as γH2Ax and 53BP1.

Summary

This study will explore the mechanistic consequences of tankyrase-dependent telomere remodelling using a comprehensive approach that combines biochemical, biophysical and cell-based studies.

LITERATURE REFERENCES
1. de Lange, T. Shelterin-Mediated Telomere Protection. *Annu. Rev. Genet.* (2018). doi:10.1146/annurev-genet-032918-021921 2. Lim, C. J. & Cech, T. R. Shaping human telomeres: from shelterin and CST complexes to telomeric chromatin organization. *Nat Rev Mol Cell Biol* 1–16 (2021). doi:10.1038/s41580-021-00328-y 3. Lim, C. J., Zaug, A. J., Kim, H. J. & Cech, T. R. Reconstitution of human shelterin complexes reveals unexpected stoichiometry and dual pathways to enhance telomerase processivity. *Nat Comms* **8,** 1075 (2017). 4. Smith, S., Giriat, S., Schmitt, A. & de Lange, T. Tankyrase, a Poly(ADP-Ribose) Polymerase at Human Telomeres. **282,** 1484–1487 (1998). 5. Smith, S. & de Lange, T. Tankyrase promotes telomere elongation in human cells. *Curr. Biol.* **10,** 1299–1302 (2000). 6. Shay, J. W. & Wright, W. E. Telomeres and telomerase: three decades of progress. *Nat. Rev. Genet.* **20,** 299–309 (2019). 7. Yin, J. *et al.* Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 15815–15820 (2005). 8. Ranes, M., Zaleska, M., Sakalas, S., Knight, R. & Guettler, S. Reconstitution of the destruction complex defines roles of AXIN polymers and APC in β-catenin capture, phosphorylation, and ubiquitylation. *Molecular Cell* **81,** 3246–3261.e11 (2021). 9. Chen, Y. *et al.* A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins. **319,** 1092–1096 (2008). 10. Schmid-Burgk, J. L., Höning, K., Ebert, T. S. & Hornung, V. CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat Comms* **7,** 12338 (2016).

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:	BSc Hons in biochemistry, molecular biology, chemistry or similar (some laboratory experience is required), scientific curiosity and a keen interest in research
Intended learning outcomes:	• Expert in protein biochemistry • Expert in mechanisms of telomere maintenance • Expert in biophysical techniques for the characterisation of proteins and protein-protein interactions • Expert in single-molecule fluorescence techniques • Expert in CRISPR-based gene editing • Expert in quantitative fluorescence microscopy • Skills in integrating numerous orthogonal approaches to investigate a biological problem
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. PhD biochemistry**
	2. protein biochemistry
	3. biophysics
	4. single-molecule fluorescence
	5. CRISPR
	6. telomere biology