Decoding telomere gene variation through saturation genome editing: from functional genomics to clinical interpretation

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General Introduction & Scope of Thesis

GENERAL INTRODUCTION

Over little more than two decades, advances in human genomics have transformed biomedical research and clinical practice. Completion of the Human Genome Project[1,2] provided the first comprehensive reference sequence of the human genome. The subsequent rapid development of next-generation sequencing (NGS) technologies has made it possible to interrogate both the coding and non-coding regions of individual genomes at scale. In clinical genetics and genomic medicine, multigene panels, exome sequencing and, increasingly, whole-genome sequencing (WGS) are now routinely used to detect germline variants associated with inherited disease predisposition. These approaches are also used to characterise somatic variation in tumours and other pathological tissues for diagnostic stratification and therapeutic guidance[3–5]. Large-scale national sequencing initiatives, such as the UK's 100,000 Genomes Project[6] and the Dutch government's implementation of WGS for cancers of unknown primary tumour site[7,8], have further demonstrated how population-scale sequencing can be integrated into healthcare delivery and have helped accelerate the adoption of clinical genomics. In principle, genomic information can enable accurate diagnosis, reveal underlying disease mechanisms, inform drug development and guide prevention and treatment strategies[9]. In practice, however, sequencing yields large numbers of variants of unknown pathogenicity, and the interpretive capacity of clinical genetics has not kept pace with the accelerating rate of variant discovery[10]. For many genes, including the telomere biology genes central to this thesis, the majority of identified variants lack robust functional or clinical evidence[11]. As a result, the major challenge has shifted: while variants can now be identified at scale, their functional and clinical interpretation remains a major bottleneck.

CLINICAL CHALLENGE: VARIANTS OF UNCERTAIN SIGNIFICANCE (VUS) IN CANCER PREDISPOSITION GENES

The rapid adoption of multigene panel testing and WGS in oncology has led to a surge in the discovery of germline variants in cancer predisposition genes[12]. At the same time, large population genetic datasets such as gnomAD[13], UK Biobank (UKBB)[14] and other population genomic studies[15–17] have catalogued millions of variants, greatly increasing variant discovery. However, for the majority of these variants, there is insufficient functional or clinical evidence to determine their clinical significance[18,19]. As a result, rare missense variants, identified through genetic testing, are most often classified as variants of uncertain significance (VUS), and in many cancer predisposition genes VUS substantially outnumber pathogenic variants[20–

[22]. VUS cannot be used to confirm or exclude a diagnosis, guide clinical surveillance, or support risk-reducing interventions. In ClinVar[23], VUS constitute a large proportion of submitted variants, and it has been reported that 20-40% of individuals undergoing hereditary disease panel testing receive at least one VUS classification, with similarly high rates reported for cancer gene panels[18,22].

Moreover, the burden of VUS is not evenly distributed across populations. Because most genomic reference and clinical sequencing datasets are still dominated by individuals of European genetic ancestry, people from under-represented groups are therefore more likely to carry rare variants for which population frequency data and prior clinical observations are limited[24–27]. As shown in data analyses from the gnomAD[13,28] and All of Us[29] cohorts, VUS rates in clinically actionable genes such as *BRCA1*, *TP53* and *PTEN* are consistently higher in non-European ancestry groups[24]. This imbalance contributes to missed or delayed diagnoses, higher rates of inconclusive clinical reports, and unequal medical healthcare across patient populations[30–32]. In hereditary cancer genetics, these effects translate into uncertain or conflicting variant interpretations that limit risk assessment, cascade testing, and surveillance decisions for families, which all together may increase patient anxiety and delay timely diagnosis[19,33].

Traditional approaches for variant interpretation integrate multiple sources of evidence, including family segregation data, population allele frequencies, evolutionary conservation, *in silico* variant effect predictors (VEPs), and targeted functional assays[34]. Modern VEPs such as AlphaMissense[35] improve prediction of missense variant effects by incorporating evolutionary and protein structure information, but are limited to protein-coding changes and cannot substitute clinical or functional validation in variant classification[36]. As a result, for many newly observed variants, particularly rare missense, non-canonical splice changes or non-coding variants, these sources of evidence are often limited or inconclusive, leaving the majority of variants classified as VUS[37,38]. Moreover, although computational predictors are widely available and scalable, they often disagree, show substantial error rates in well-characterised disease genes, and can overestimate pathogenicity for variants with limited functional impact, reducing their reliability as standalone clinical evidence[36,39–41]. Although the American College of Medical Genetics and Genomics (ACMG) / Association for Molecular Pathology (AMP) guidelines[34] recognise well-validated gene-specific functional studies as ‘strong’ evidence for pathogenicity or benignity (PS3/BS3), such assays are labour- and time-

intensive, typically only initiated after a variant has emerged in a clinical context, and therefore cannot feasibly keep pace with the rapid discovery of novel variants[42]. As a result, the gap between variant detection and variant interpretation continues to widen, underscoring the need for scalable experimental approaches capable of generating functional evidence at saturation depth[42].

MULTIPLEXED ASSAYS OF VARIANT EFFECT: A SCALABLE SOLUTION

Efforts to close the gap between variant discovery and interpretation have increasingly turned to multiplexed assays of variant effect (MAVE). These high-throughput experiments have the capacity to measure the functional consequences of thousands of variants in a single pooled assay[42,43]. In a typical MAVE, a designed library of variants, for example all possible single-nucleotide substitutions, across the coding sequence of a gene, is introduced into a suitable model system. Variants are then subjected to functional selection, such as cell growth[44–60], drug response[54,61,62], reporter activity measuring transcriptional activity or protein-protein interactions[63–65], protein abundance[59,66–69], splicing[70] or other molecular phenotypes[67,71]. In growth-based assays, deep sequencing quantifies changes in variant frequency after selection, whereas in protein abundance, reporter, or splicing assays, variants are grouped based on the measured phenotype and sequencing is used to identify which variants fall into each group. The MAVE output is a ‘variant effect map’, which represents a dense data frame of scores, that links each tested variant to a quantitative readout of its impact on gene-associated molecular function[72].

MAVE can be implemented across a wide range of systems, from simple organisms such as phage[73], bacteria[74] and yeast[48,75] to immortalised human cells and stem-cell–derived models[76–78]. For protein-coding variants, commonly used approaches include deep mutational scanning (DMS) to assess protein function, complementation assays in yeast or human cells, and assays that quantify protein abundance or stability, such as variant abundance by massively parallel sequencing (VAMP-seq)[69]. Non-coding variants are often assessed using reporter-based approaches or splicing assays that test regulatory and splice-associated sequences[70,79,80]. Across all platforms, assay design is defined by how variants are introduced (for example cDNA libraries versus genome editing libraries), the cellular context, and the phenotypic readout. These choices influence which classes of variant effect are most readily captured, such as loss-of-function (LoF), gain-of-function (GoF), or splicing and regulatory defects, while the same gene may exhibit different mechanisms depending on assay context, and determine how directly the resulting data can inform clinical variant interpretation[50,72].

Among these MAVE technologies CRISPR-based saturation mutagenesis, particularly Saturation Genome Editing (SGE), has emerged as a powerful approach for studying disease-associated genes[45,81]. In SGE, CRISPR-Cas9 and homology-directed repair (HDR) are used to introduce a library of single-nucleotide substitutions (and other variant classes), and often clinically observed small insertions or deletions, into an endogenous gene whose function can be coupled to cellular fitness in an appropriate cell line. Edited cells are then cultured under conditions in which gene function is linked to survival or proliferation, and high-throughput sequencing of the targeted region at baseline and after a selected time window is used to infer a fitness score for each variant. Variants that disrupt gene function become depleted from the cell population, whereas neutral or tolerated variants remain stable. Because SGE operates at the native genomic locus, it preserves endogenous transcriptional regulation, splicing, mRNA turnover and chromatin context, avoiding some of the artefacts of cDNA overexpression systems[44,45,52].

The first large-scale SGE map of *BRCA1* functional domains[45] showed that functional scores generated in the native genomic locus correlate strongly with clinical classifications and enabled reinterpretation of many previously unclassified missense variants. Since then, SGE and related CRISPR-based MAVE approaches have been applied to an expanding set of clinically relevant genes, including, *BAP1*[44], *BRCA2*[47], *CARD11*[50], *DDX3X*[52], *RAD51C*[57], and *VHL*[58] with ongoing technical advances enabling near-complete interrogation of all possible single-nucleotide coding variants in selected regions. Across platforms, MAVE are now estimated to have generated functional measurements for over ten million variants, offering a powerful, though still evolving, experimental framework for large-scale variant interpretation[82].

In parallel with advances in MAVE technology, efforts are underway to establish how large-scale functional data should be interpreted in a clinical context. Variant classification frameworks such as the ACMG/AMP guidelines were developed around low-throughput functional assays, and did not initially account for approaches such as MAVE[34,83,84]. More recently, clinical genomics initiatives have begun to explore how MAVE data could be evaluated for reliability, calibrated against well-characterised clinical variants, and incorporated into existing ACMG/AMP evidence categories[84–86]. Although this integration is still ongoing and methodologically complex, MAVEs are increasingly viewed as a promising source of functional evidence for variant interpretation, particularly for variants that lack population or segregation data[24].

These developments support the view that generating high-quality MAVE datasets for clinically important genes, including telomere biology genes, should be a priority both for improving clinical variant interpretation and for addressing inequities in genomic medicine[24,87]. In this context, this thesis applies SGE to generate variant effect maps for two key telomere-associated genes *POT1* and *TINF2* and explores how these data can be integrated with telomere length measurements, population-scale datasets and clinical phenotypes.

TELOMERE BIOLOGY AND TELOMERE-ASSOCIATED DISORDERS

While MAVEs are broadly applicable, this thesis focuses on their use in telomere biology, and in particular on the shelterin genes implicated in hereditary cancer and telomere syndromes.

TELOMERE STRUCTURE AND FUNCTION

Telomeres are specialised nucleoprotein structures that cap the ends of linear chromosomes and consist of tandem hexanucleotide (TTAGGG) repeats bound by a dedicated set of proteins collectively referred to as the shelterin complex[88]. The telomeric tract consists primarily of double-stranded telomeric repeats and terminates in a single-stranded 3′ overhang that can loop back into the duplex region, forming a higher-order T-loop structure with an associated displacement loop (D-loop)[89,90]. This architecture, stabilised by shelterin, conceals the chromosome terminus, and prevents it from being misidentified as a DNA double-strand break (DSB)[91]. By suppressing ataxia-telangiectasia mutated (ATM)- and ataxia-telangiectasia and Rad3-related (ATR)-mediated signalling and thereby blocking inappropriate DNA damage response (DDR) pathways, telomeres play a central role in maintaining chromosome stability through successive cell divisions.

THE SHELTERIN COMPLEX

The shelterin complex is composed of six core proteins with distinct DNA-binding and regulatory roles (**Figure 1**). TRF1 (*TERF1*) and TRF2 (*TERF2*) bind the double-stranded telomeric repeats; RAP1 (*TERF2IP*) associates with TRF2; TIN2 (*TINF2*) acts as a central scaffold that links TRF1 and TRF2 to the TPP1 (*ACD*) - POT1 heterodimer, and POT1 binds the single-stranded 3′ overhang via its oligonucleotide/oligosaccharide-binding (OB) fold domains[92,93,88]. Through these coordinated interactions, shelterin maintains the T-loop

configuration, protects chromosome ends from aberrant DNA damage responses, and regulates telomere length homeostasis by controlling access of telomerase to the chromosome terminus. TRF1 and TRF2 mainly restrict telomerase access to double-stranded telomeric DNA, while POT1 in complex with TPP1 regulates access at the single-strand level and coordinates post-replicative C-strand fill-in via interaction with the CTC1-STN1-TEN1 (CST)-DNA polymerase α complex. Through its telomerase essential (TEL) patch, TPP1 functions as the primary interface for telomerase recruitment and enhances enzyme processivity, establishing it as the principal positive regulator of telomere elongation within the shelterin system[94]. Overall, shelterin performs two inseparable functions: (i) biochemical insulation of chromosome ends from DNA damage sensors, and (ii) fine control of telomerase activity and overhang processing[88,92,93].

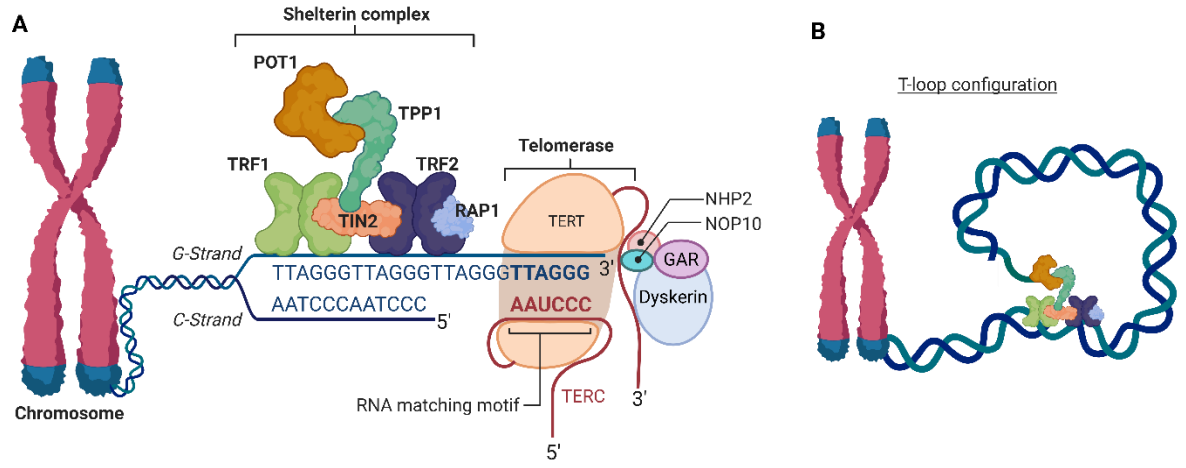

Figure 1: Organisation of the telomere, shelterin complex, and telomerase.
A) Schematic representation of a human chromosome end showing the telomeric double-stranded region and single-stranded 3′ overhang. The shelterin complex (POT1, TPP1, TIN2, TRF1, TRF2 and RAP1) binds telomeric DNA to protect chromosome ends and regulate telomerase access. Telomerase, composed of the catalytic subunit TERT, the RNA template TERC, and associated factors, including dyskerin, extends telomeric repeats. **B)** Formation of the T-loop in which the single-stranded 3′ overhang is sequestered to prevent inappropriate DNA damage responses (DDR). POT1 (orange) is the only shelterin component that binds the single-stranded 3’ overhang.

TELOMERASE

Telomerase is a ribonucleoprotein reverse transcriptase composed of the catalytic subunit TERT and an RNA template (TERC), together with accessory proteins including dyskerin, NOP10[95], NHP2[96] and TCAB1[97] that assemble the functional holoenzyme[98]. It catalyses the addition of TTAGGG repeats to chromosome ends, with its recruitment, processivity and telomere selectivity governed by shelterin-mediated regulation. Telomerase action is temporally restricted to late S phase and is limited to a subset of chromosome ends in each cell cycle, thereby maintaining telomere length within a defined range rather than equalising

all telomeres[99]. In healthy human tissue, telomerase is active in embryonic cells, the germline and selected adult stem-cell compartments, but transcriptionally silenced in most somatic cells[100]. Consequently, telomerase activity determines whether telomeres shorten progressively or are maintained over time.

Approximately 85-90% of cancers reactivate telomerase, most often through TERT promoter mutation, gene amplification or epigenetic derepression[101]. This enables continued cell proliferation and telomere elongation beyond the normal replicative limit. However, telomerase reactivation alone is not sufficient to confer cancer risk; rather, dysregulation of telomerase access by shelterin, particularly involving POT1 and TPP1, can drive pathological telomere elongation and cancer predisposition[102].

ALTERNATIVE LENGTHENING OF TELOMERES (ALT)

In addition to telomerase, telomere length can be maintained by the alternative lengthening of telomeres (ALT), a telomerase-independent mechanism that relies on homologous recombination (HR) rather than reverse transcription[103,104]. In ALT-positive cells, telomeres are extended through recombination-mediated copying of telomeric sequence from other chromosome ends or extrachromosomal telomeric DNA, resulting in a highly heterogeneous telomere length distribution in which individual cells harbour both excessively long and critically short telomeres. ALT is observed predominantly in cancer, particularly in sarcomas and subsets of gliomas, and accounts for approximately 5-15% of tumours[105]. This pathway is strongly associated with loss of the chromatin regulators ATRX or DAXX, which disrupt telomeric chromatin and facilitate HR-based elongation. ALT-positive tumours are often highly aggressive and genetically unstable, reflecting their dependence on aberrant DNA repair processes.

TELOMERE REPLICATION AND SHORTENING

Like the rest of the genome, telomeres are replicated by the conventional DNA replication machinery. However, replication is intrinsically incomplete at chromosome termini because the final RNA primer on the lagging C-strand (the strand complementary to the 3’ G-rich overhang) cannot be positioned at the extreme chromosome end. Removal of this primer leaves a terminal gap that cannot be filled by DNA polymerase. This limitation, known as the end-replication problem, which results in progressive telomere erosion with each cell division[106].

This asymmetry arises from the distinct modes of strand synthesis during DNA replication: the G-rich leading strand (G-strand) is synthesised continuously, whereas the C-rich lagging strand (C-strand) is synthesised discontinuously from multiple RNA primers. Because the final RNA primer on the lagging strand cannot be positioned at the chromosome terminus, its removal leaves the C-strand progressively shorter after each round of replication. To generate a functional chromosome end, telomeres undergo post-replicative processing that produces a single-stranded 3′ G-strand overhang, which is required for POT1-TPP1 binding and T-loop formation[107]. This overhang is generated by nucleolytic resection of the C-strand, mediated primarily by the TRF2-associated exonuclease Apollo, with additional contributions from EXO1 and MRE11[108]. Although essential for telomere protection, this processing inherently removes DNA from chromosome ends and therefore contributes further to telomere shortening. Following overhang formation, the double-stranded telomeric DNA region is partially restored by the CST complex, which recruits DNA polymerase α–primase to resynthesise the C-strand[109]. In parallel, telomerase may extend the G-strand. Together, CST-mediated C-strand fill-in and telomerase-dependent G-strand extension act as a coupled mechanism to restore telomere structure after replication.

Telomeric DNA is thus lost through incomplete replication of the C-strand and nucleolytic resection. In germline and embryonic stem cells, telomerase activity compensates for this erosion. In contrast, most somatic cells express insufficient telomerase, resulting in progressive telomere shortening of approximately 50-200 base pairs (bp) per division and eventual replicative senescence[108].

TELOMERE DYSFUNCTION AS A FORK BETWEEN DEGENERATIVE DISORDERS AND CANCER PREDISPOSITION

When telomeres shorten beyond a critical threshold in normal somatic cells, there are insufficient telomeric repeats to support full shelterin binding. Loss of adequate TRF1/TRF2 binding to double-stranded telomeric repeats, together with incomplete protection of the single-stranded 3’ overhang by POT1, expose chromosome ends to canonical DNA damage sensors, including the ATM and ATR kinases, the Ku70/80 heterodimer, and replication protein A (RPA)[90,110]. This telomere deprotection elicits a persistent DDR, leading to activation and stabilisation of p53, transcriptional induction of p21, engagement of the p16 (INK4a) pathway, and irreversible cell-cycle arrest in the form of replicative senescence[111–114]. This core tumour-suppressive mechanism prevents the propagation of cells with unstable chromosome ends but also limits tissue regenerative capacity by restricting stem- and progenitor-cell proliferation.

In contrast, pathogenic disruption of shelterin or other telomere maintenance components can induce telomere deprotection in contexts where cell-cycle checkpoints are bypassed or eventually eroded, allowing damaged chromosome ends to persist. Under these conditions, chromosome ends become functionally uncapped, enabling end-to-end fusions and breakage-fusion-bridge (BFB) cycles that drive genomic instability. Sustained telomere dysfunction can culminate in telomere crisis, characterized by widespread chromosomal instability, including chromothripsis, kataegis, and tetraploidy, which, if not resolved by senescence or apoptosis, can promote malignant transformation[115].

Observations in human cancer cells suggest a functional lower limit for telomere length of approximately 77 bp, below which telomere-telomere fusions are observed[116]. Importantly, even a single critically short or unprotected chromosome end is sufficient to activate a DDR and impose cell-cycle arrest, regardless of the average telomere length[117].

The biological consequences of telomere dysfunction are not determined solely by telomere length, but by how specific perturbations affect the dual functions of the shelterin complex: (i) protection of chromosome ends from DDR activation, and (ii) regulation of telomere replication and telomerase access[118,119]. As highlighted in recent work[119], shelterin components, particularly POT1 and TIN2, operate at the interface of these processes by coordinating end protection, control of ATR signalling, recruitment of telomerase via TPP1, and regulation of C-strand fill-in through CST–Polα/primase.

Disruption of these functions can therefore produce divergent telomere phenotypes[102]. Variants that impair telomere replication, end processing, or structural integrity, such as defective POT1-mediated C-strand fill-in or destabilisation of the TIN2-centred shelterin scaffold, lead to telomere instability, stochastic truncation, and progressive shortening, ultimately driving stem-cell exhaustion and degenerative disease[102,118,119]. Conversely, variants that selectively weaken shelterin-mediated restriction of telomerase access, while preserving sufficient end protection to avoid constitutive DDR activation, permit excessive telomere elongation[102,119]. In this context, telomeres remain functionally capped but extend beyond their normal homeostatic range, conferring increased replicative capacity and thereby predisposing cells to malignant transformation[102,118].

Thus, telomere pathology is determined not by telomere length alone, but by the functional balance between telomere end protection, DNA replication, and elongation. Disruption of this balance creates a mechanistic fork between two pathological trajectories: premature replicative arrest and organ failure arising from impaired telomere maintenance, and pathological extension of cellular lifespan with increased oncogenic risk when telomere length

control is relaxed, underpinning the spectrum of telomere biology disorders and long-telomere cancer predisposition syndromes, respectively.

SHORT TELOMERE SYNDROMES (TELOMERE BIOLOGY DISORDERS)

Telomere Biology Disorders (TBDs) arise from progressive telomere shortening or instability caused by inherited defects in telomerase activity, telomere replication, or shelterin function[102,120,121]. These defects impair telomere maintenance, leading to accelerated telomere attrition per cell division. As telomeres reach critically short lengths, chromosome ends progressively lose effective shelterin-mediated protection, triggering chronic DDR signalling and stem- and progenitor-cell exhaustion. Because telomere erosion is most consequential in tissues with high proliferative demand, organs such as the bone marrow, lung, and liver are affected earliest, giving rise to the degenerative phenotype characteristic of TBDs.

Collectively, TBDs encompass a spectrum of disorders defined by impaired stem-cell function, reduced cellular replicative capacity, and increased cancer susceptibility[122]. These include dyskeratosis congenita (DC)[123,124], Hoyeraal-Hreidarsson[125], Revesz syndrome[126], familial pulmonary fibrosis[127,128], and inherited bone marrow failure syndromes[129]. Pathogenic variants have been identified in genes encoding telomerase components and biogenesis factors (*TERT*, *TERC*, *DKC1*), DNA replication and repair factors (*RTEL1* and CST complex genes), and shelterin components, including *TINF2* and *POT1*[130,152–154]. *TINF2*-associated disease is particularly notable for its severity: truncating variants in the C-terminal “DC patch”[131,132] destabilise shelterin globally, resulting in profound telomere shortening despite preserved telomerase activity. This observation demonstrates that telomere end protection is as critical as telomere elongation in maintaining telomere integrity[88].

Clinically, patients with TBD typically exhibit very short telomeres, often below the 1st percentile for age, and may present with bone marrow failure, pulmonary fibrosis, liver disease, immunodeficiency, and the characteristic mucocutaneous features of DC[133]. Individuals are also at increased risk of malignancies, particularly myelodysplastic syndrome, acute myeloid leukaemia, and head and neck squamous cell carcinoma, establishing telomere dysfunction as a direct cause of inherited cancer predisposition [134–136]. A hallmark of TBDs is variable expressivity: the same pathogenic variant may cause severe childhood disease in one individual yet isolated adult-onset pulmonary fibrosis in another[121,128,137]. Diagnosis typically relies on telomere length measurement (e.g. flow-FISH), often in combination with genetic testing, and has important implications for management, including modified hematopoietic stem-cell transplantation strategies and enhanced cancer and organ-specific surveillance[138].

LONG TELOMERE SYNDROMES (CANCER PREDISPOSITION WITH LONG TELOMERES)

In contrast, Cancer Predisposition with Long Telomeres (CPLT) arises from germline variants, most commonly in shelterin components such as *POT1* and *TINF2*, that perturb shelterin-mediated control of telomere length and telomerase access. These variants permit excessive telomere lengthening without triggering a robust DDR, allowing cells to evade senescence and acquire an extended replicative lifespan, thereby increasing the cumulative opportunity for oncogenic mutations and clonal evolution[102].

The best characterised example of CPLT is the *POT1* tumour predisposition syndrome (POT1-TPD), which exemplifies how disruption of shelterin-mediated telomere regulation leads to inherited cancer susceptibility through telomere overextension[139,140]. Clinically, *POT1* LoF germline variants predispose to melanoma[141], chronic lymphocytic leukaemia (CLL)[142], glioma[142,143] and angiosarcoma[143]. Recurrent somatic *POT1* mutations have also been reported in haematological malignancies[144–146], further supporting *POT1* is a key tumour suppressor at chromosome ends[140]. Current evidence suggests that germline CPLT *POT1* variants promote telomere lengthening by permitting telomerase-dependent telomere maintenance while preserving sufficient telomere end protection to avoid a strong DDR, thereby extending cellular replicative lifespan[141,147,148]. Although *POT1* has been reported to both enhance telomerase processivity[149] and negatively regulate telomerase catalytic activity[150] in different experimental contexts, the prevailing view is that haploinsufficiency or partial loss of POT1 function relaxes telomere length control rather than causing telomere attrition, resulting in long telomeres that support clonal persistence and cancer predisposition[151]. By contrast, *POT1* variants associated with TBDs are linked to telomere loss and progressive shortening[152–154], underscoring distinct telomere length outcomes associated with different classes of POT1 dysfunction.

Comparable long-telomere phenotypes have also been observed in families carrying pathogenic variants in other shelterin components, including *ACD*[155,156], *TERF2IP*[155,156] and *TERF1*[157], as well as in specific *TINF2* alleles that are distinct from those causing TBDs[102,158]. Long-telomere, cancer-associated *TINF2* variants are typically truncating haploinsufficient alleles[159–161], and have been identified in patients with melanoma[159], thyroid carcinoma[160] and sarcoma[159].

Together, these observations indicate that telomere length outcomes strongly depend on the functional domain affected and cellular context rather than on variant class or zygosity alone. Given that variants in *POT1* and *TINF2* can result in either excessive telomere elongation or

severe telomere shortening, these genes are particularly important to study for understanding how altered telomere regulation contributes to CPLT and TBD.

CLINICAL RELEVANCE AND RATIONALE FOR FUNCTIONAL MAPPING

For clinicians, the biological complexity of telomere maintenance creates major challenges for variant interpretation. As illustrated in this chapter, missense changes in shelterin genes can be benign, cause telomere shortening and stem-cell failure, or predispose to cancer through excessive telomere elongation, outcomes that are rarely distinguishable using computational tools or limited case evidence. As hereditary cancer and telomere disorder panels increasingly include shelterin genes, laboratories are identifying growing numbers of VUS in *POT1*, *TINF2*, *ACD, TERT* and related genes[19,20,147,160], delaying diagnosis, complicating management decisions, and hindering genetic counselling.

From an oncological perspective, failure to establish an accurate molecular diagnosis can result in patients missing appropriate surveillance, receiving poorly tolerated cytotoxic therapies, and leaving familial risk unrecognised. Conversely, accurate identification of pathogenic variants enables cancer risk stratification, informed surveillance, treatment modification, and predictive testing of relatives.

This thesis addresses these challenges by applying SGE to *POT1* and *TINF2* to generate comprehensive variant-effect maps. These two genes were prioritised among shelterin components because they are among the most frequently implicated in human disease, with recurrent germline variants identified in both TBDs[131,132,152–154,158] and cancer predisposition syndromes[141–148,159–161], and because they occupy central and functionally distinct positions within the shelterin complex (POT1 at the single-stranded overhang and TIN2 as a scaffold linking double- and single-stranded binding components)[88,92,93], making them particularly informative for dissecting mechanisms of telomere dysfunction.

The SGE maps systematically link nearly all possible coding variants to cellular phenotypes, primarily cell fitness effects, and are subsequently integrated with orthogonal telomere-related data, including DDR signalling, and telomere length measurements, as well as clinical evidence such as family segregation and population-level cancer risk. Together, this work aims to provide a scalable, experimentally grounded evidence for improving variant interpretation in telomere biology genes, with the potential to improve diagnostic precision in telomere-mediated disease and cancer predisposition.

THESIS OUTLINE AND OBJECTIVES

This thesis aims to define how variation in the telomere biology genes *POT1* and *TINF2* affects gene function, telomere regulation, and cancer risk, and to evaluate SGE as a practical approach for resolving VUS in these genes. **Chapter 2** presents a state-of-the-art review of CRISPR-based endogenous MAVEs, comparing SGE, base and prime editing, and discussing how different editing strategies and readouts can be combined to link variant function to disease mechanisms and clinical translation across genetic disorders. **Chapter 3** describes the adaptation and optimisation of a robust SGE workflow in a haploid human cell system, including library design, editing strategies and quantitative readouts that enable reproducible scoring of tens of thousands of variants. Using this platform, **Chapter 4** generates the first comprehensive functional map of *POT1*, capturing ~97% of all possible coding substitutions and revealing domain-specific sensitivity, particularly within the OB fold DNA-binding regions. It then integrates and validates SGE classifications through telomere length and DDR readouts, large-scale UKBB analyses, clinical cancer cohorts, and the ACMG/AMP framework, demonstrating how functional effect size predicts telomere length, cancer risk, and variant pathogenicity. **Chapter 5** extends SGE to *TINF2,* interrogating how germline variants in key TIN2 shelterin domains perturb telomere regulation and contribute to cancer risk in long-telomere, cancer-prone families. By integrating SGE data with clinical and pedigree information, this chapter refines the emerging *TINF2*-associated tumour spectrum and evaluates the utility of SGE for guiding variant interpretation and clinical surveillance. **Chapter 6** applies these insights to a population-based melanoma case-control study, combining *POT1* sequencing, SGE functional scores, telomere-binding assays and molecular dynamics simulations to assess the contribution of *POT1* variation to melanoma risk in the general population. **Chapter 7** examines *POT1* genetic testing in Swedish melanoma-prone families and shows how SGE-derived functional evidence informs interpretation of variants within complex pedigrees that include early-onset disease, multiple primaries and phenocopies. Together, these chapters integrate functional genomics with genetic and population data to improve the interpretation of variation in the telomere biology genes *POT1* and *TINF2* and to evaluate the contribution of SGE to hereditary cancer genetics. Finally, **Chapter 8** integrates the findings of this thesis in the context of clinical variant interpretation and hereditary cancer genetics, discusses methodological limitations, and outlines future directions for applying SGE to variant interpretation.

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