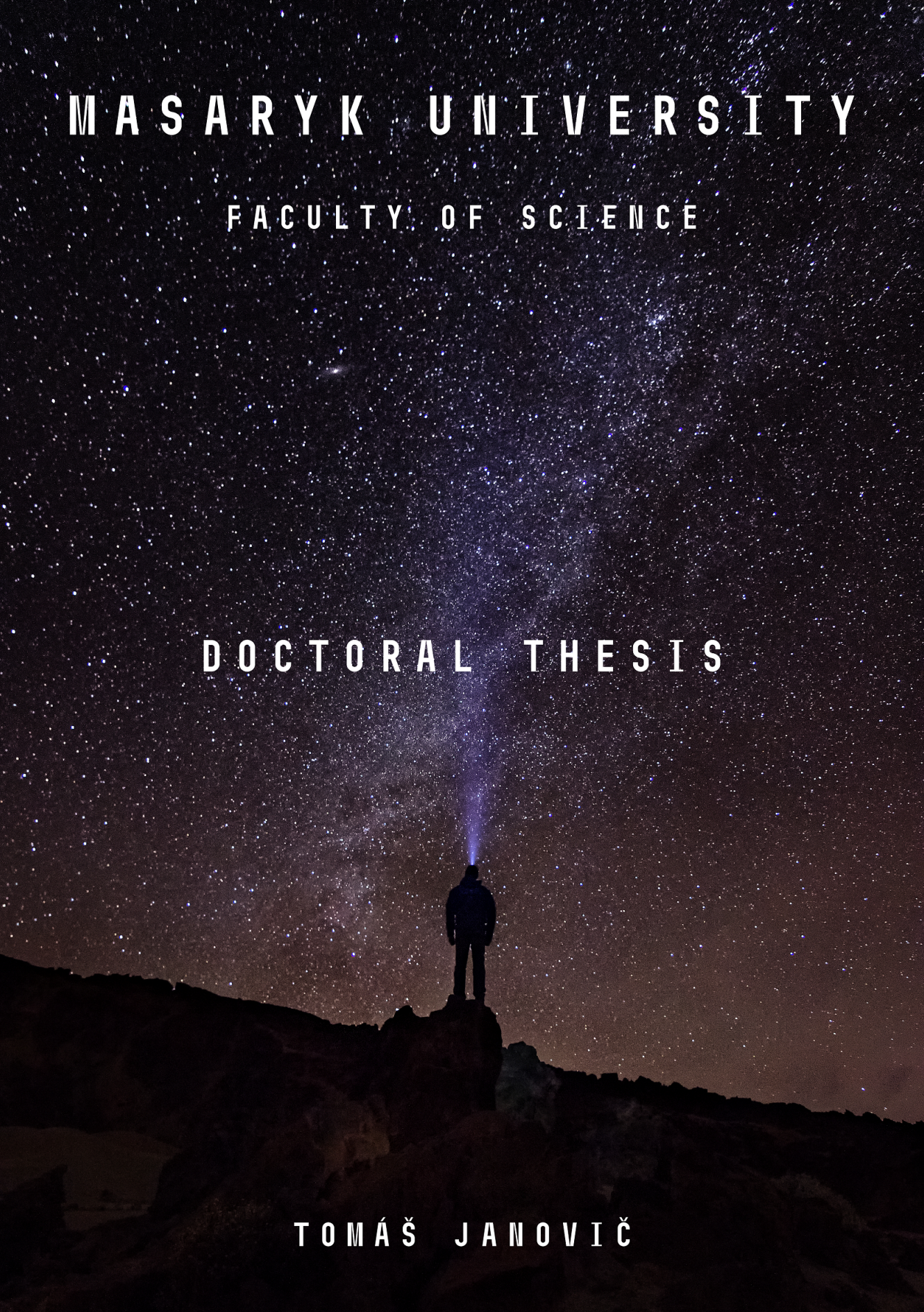


A person stands on a dark, rocky outcrop in the foreground, silhouetted against a vast, starry night sky. The person is holding a flashlight, which casts a bright beam of light upwards, illuminating the Milky Way galaxy. The sky is filled with countless stars and the dense band of the Milky Way stretches across the upper half of the image. The overall mood is one of exploration and discovery.

MASARYK UNIVERSITY

FACULTY OF SCIENCE

DOCTORAL THESIS

TOMÁŠ JANOVÍČ

Human shelterin assembly and transcription regulation

Doctoral Thesis

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Supervisor: Assoc. Prof. Ctirad Hofr, Ph.D.

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Abstract

Telomeres provide the protective cap to the ends of linear chromosomes. The regulation of telomere length is essential in maintaining genome stability. Impairing the telomere maintenance leads to dysfunctional telomeres, resulting in telomere shortening associated with diseases such as bone marrow failure, premature aging, and cancer. Telomere maintenance is dependent on strict coordination between telomeric protein complexes, telomerase, and telomeric DNA. Shelterin represents the major protein complex that binds telomeric DNA with a high affinity and sequence specificity. Moreover, shelterin directly recruits numerous telomere-associated factors, including telomerase, and is responsible for shaping the telomeric structure and guarding telomeres against DNA damage response machinery. The assembly and cooperation of human shelterin proteins can regulate the functions of the whole complex. Therefore, complex formation through dynamic interactions between shelterin subunits is essential in telomere maintenance.

To elucidate the molecular mechanism of human shelterin assembly, we focused on the telomeric repeat binding factors TRF1 and TRF2, which together with TIN2 (TERF1-interacting nuclear factor 2) form the core of the shelterin complex. We applied a single-molecule approach based on fluorescence cross-correlation spectroscopy (FCCS) to monitor the spontaneous intensity fluctuations as the differently fluorescently labeled TRF1/TRF2 and TIN2 diffuse through the confocal volume of a microscope. The cross-correlation analysis of measured intensity traces enabled us to understand the TRF1-TIN2-TRF2 complex formation. We found that TRF1 can effectively replace TRF2 bound to TIN2. In contrast, TRF2 had no evident effect on the TRF1-TIN2 complex. Furthermore, we assessed the effect of shelterin factor TPP1 (adrenocortical dysplasia protein homolog) in promoting whole shelterin complexation. Strikingly, TPP1 addition expanded the TIN2 binding capacity and allowed TIN2 to accommodate both TRF1 and TRF2 simultaneously. We proposed a model describing the assembly of human shelterin core subunits in solution. We highlighted the TPP1 binding to TIN2 as an essential step in stable shelterin formation. Moreover, we suggested

the TPP1-TIN2 interaction as the potential weakest point in shelterin assembly, hence the critical step in telomere maintenance.

The thesis outcomes contribute to understanding human shelterin assembly and describe the role of TPP1 in promoting the whole shelterin complexation. The obtained findings address the cooperation of full-length telomeric proteins essential in telomere maintenance at the single-molecule level. In addition, the FCCS-based single-molecule platform served for methodologically related studies, where we characterized the practical impacts of deuterated water in fluorescence-based methods.

Abstrakt

Telomery tvoří ochrannou část konců lineárních chromozomů. Regulace délky telomer je nezbytná pro zajištění stability genomu. Narušení udržování telomer vede k jejich nefunkčnosti, což má za následek zkracování telomer spojené s nemocemi, jako je syndrom selhání kostní dřeně, předčasné stárnutí a rakovina. Udržování telomer je závislé na přísné koordinaci mezi telomerovými proteinovými komplexy, telomerázou a telomerovou DNA. Shelterin představuje hlavní proteinový komplex, který váže telomerovou DNA s vysokou afinitou a sekvenční specifitou. Shelterin rovněž rekrutuje řadu telomerových faktorů, včetně telomerázy, a je zodpovědný za utváření telomerové struktury a ochranu telomer před opravami při poškození DNA. Seskupení a vzájemná koordinace lidských proteinů shelterinu může regulovat funkce celého komplexu. Tvorba komplexu prostřednictvím dynamických interakcí mezi podjednotkami shelterinu je proto pro udržování telomer zcela klíčová.

Abychom objasnili molekulární mechanismus formace lidského shelterinu, zaměřili jsme se na faktory TRF1 a TRF2 (telomeric repeat binding factor 1 a 2), které spolu s TIN2 (TERF1-interacting nuclear factor 2) proteinem tvoří jádro shelterinového komplexu. Aplikovali jsme "single-molecule" přístup založený na fluorescenční kros-korelační spektroskopii (FCCS) ke sledování spontánních fluktuací intenzity při difúzi různě fluorescenčně značených TRF1/TRF2 a TIN2 proteinů konfokálním objemem mikroskopu. Kros-korelační analýza naměřených signálů intenzit nám umožnila objasnit tvorbu komplexu TRF1-TIN2-TRF2. Zjistili jsme, že TRF1 faktor může efektivně nahradit faktor TRF2 navázaný na TIN2 podjednotku. Naproti tomu faktor TRF2 neměl na komplex TRF1-TIN2 žádný zřetelný vliv. Dále jsme hodnotili vliv shelterinového faktoru TPP1 (adrenocortical dysplasia protein homolog) na podporu komplexace celého shelterinu. K našemu očekávání, přidavek proteinu TPP1 rozšířil vazebnou kapacitu podjednotky TIN2 a umožnil, aby navázal současně faktory TRF1 i TRF2. Navrhli jsme model popisující komplekci klíčových podjednotek lidského shelterinu v roztoku. Vyzdvihli jsme vazbu proteinu TPP1 na TIN2 podjednotku jako esenciální krok při formování stabilního shelterinu. Následně jsme navrhli interakci TPP1-TIN2 jako

potenciálně nejslabší místo při vytváření shelterinu a tedy i při udržování telomer.

Výsledky práce přispívají k objasnění mechanismu sestavování lidského shelterinu a popisují úlohu proteinu TPP1 při zprostředkování komplexace shelterinu. Získané poznatky se zabývají kooperací telomerových proteinů o plné délce, které jsou nezbytné pro udržování telomer, na "single-molecule" úrovni. Mimoto posloužila "single-molecule" platforma založená na FCCS k metodicky příbuzným studiím, kde jsme charakterizovali praktické dopady deuterované vody v metodách založených na fluorescenci.

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Declaration

I hereby declare that I worked on this thesis independently, and I used only the literature included in the list of references.

Brno, 21.3.2022

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Tomáš Janovič

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ABBREVIATIONS

53BP1	P53-Binding Protein 1
ALT	Alternative Lengthening of Telomeres
A-NHEJ	Alternative Non-Homologous End-Joining
APBs	ALT-associated PML Bodies
ATM	Ataxia–Telangiectasia Mutated serine/threonine kinase
ATR	Ataxia–Telangiectasia and Rad3-related protein
BIR	Break-Induced Replication
BLM	Bloom Syndrome
BRCT	BRCA1 C-Terminal
C-NHEJ	Classical Non-Homologous End-Joining
Cryo-EM	Cryo-Electron Microscopy
CPM	Counts per second Per Molecule
CST	CTC1–STN1–TEN1
CTE	Carboxy-Terminal Extension
DAPI	4', 6-DiAmidino-2-PhenylIndole
DC	Dyskeratosis Congenita
DDR	DNA Damage Response
DLS	Dynamic Light Scattering
EBV	Epstein-Barr Virus
ECTRs	Extrachromosomal Telomeric Repeats
FA	Fluorescence Anisotropy
FCS	Fluorescence Correlation Spectroscopy
FCCS	Fluorescence Cross-Correlation Spectroscopy
FLIM	Fluorescence Lifetime Microscopy
GAR1	H/ACA Ribonucleoprotein Complex Subunit 1
GQ	G-Quadruplex
HBV	Hepatitis B Virus
HBx	HBV x
HBZ	HTLV-1 bZIP

HDR	Homology-Directed Repair
HR	Homologous Recombination
HTLV-1	Human T-cell Lymphotropic Virus 1
IF	ImmunoFluorescence
IR	InfraRed
ITRs	Interstitial Telomeric Repeats
LLPS	Liquid-Liquid Phase Separation
MCPH1	Microcephalin 1
MRN	Mre11, Rad50, Nbs1 complex
MST	MicroScale Thermophoresis
NHEJ	Non-Homologous End-Joining
NHP2	H/ACA Ribonucleoprotein Complex Subunit 2
NOP10	H/ACA Ribonucleoprotein Complex Subunit 3
OB	Oligonucleotide/Oligosaccharide Binding
PCNA	Proliferating Cell Nuclear Antigen
POT1	Protection of Telomeres 1
PML	Promyelocytic Leukemia Protein
PNUTS	Phosphatase Nuclear Targeting Subunit
PTM	Post-Translational Modification
Rap1	Repressor Activator Protein 1
REST	RE1-Silencing Transcription factor
RCT	Rap1 C-Terminal
RFC	Replication Factor C
RIF1	Rap1-Interacting Factor 1
RNP	RiboNucleoProtein
RPA	Replication Protein A
Rta	Replication and Transcription Activator
SDS-PAGE	Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
SNR	Signal-to-Noise Ratio
TBD	TPP1 Binding Domain
TBM	TIN2 Binding Motif
TCAB1	Telomerase Cajal Body Protein 1
TEN	Telomerase Essential N-terminal
TERT	Telomerase Reverse Transcriptase
TIN2	TERF1-Interacting Nuclear Factor 2
TPP1	Adrenocortical Dysplasia Protein Homolog
TR	Telomerase RNA
TRF1	Telomeric Repeat Binding Factor 1

TRF2	Telomeric Repeat Binding Factor 2
TRFH	Telomeric Repeat Factors Homology
T-SCEs	Telomere-Sister Chromatid Exchanges
vTRs	viral Transcriptional Regulators

1 TELOMERES

Nucleoprotein structures at the end of eukaryotic chromosomes are called telomeres. In the late 1930s, Herman Müller was the first who discovered telomeres during his study of chromosome behavior in flies. He found that the ends of the chromosomes after irradiation with X-rays did not present any alterations, which he suggested to be thanks to the terminal protective cap. Müller finally called the telomere from the Greek terms *telos* (end) and *meros* (part) [1]. Soon after, Barbara McClintock supported Müller's discovery when she described the fusion of broken chromosome ends, resulting in the formation of dicentric chromosomes in corn [2]. Müller and McClintock assumed that telomeres present a unique structure and are essential in chromosome stability and integrity. Their findings were addressed before DNA was recognized as genetic material.

In the early 1960s, Leonard Hayflick discovered that cultured human fibroblasts have a limited replication capacity and ruled out Alexis Carrel's theory about normal human cell indefinite proliferation. He broadened the discovery by finding that only cancer cells can undergo an unlimited number of replications. In contrast normal cells have a mechanism that controls the number of cell divisions after reaching a maximum known as a Hayflick limit [3].

The elucidation of DNA structure brings scientists new insights into how genetic material could be replicated and maintained across generations of organisms. In the early 1970s, James Watson identified the end replication problem. He postulated that cells could not completely replicate the very ends of their DNA due to the incomplete synthesis of lagging-strand mechanisms [4]. Shortly after, Alexei Olovnikov linked the end replication problem with cellular senescence. He proposed a counting mechanism model describing why the end replication problem is the reason for telomere shortening after each cell division [5]. Watson and Olovnikov believed that cells possess a mechanism that controls and maintains the telomere length during DNA replication.

In 1978, Elizabeth Blackburn described telomeres from *Tetrahymena thermophila* and identified the first telomere sequence consisting of GC-rich hexanu-

cleotide tandem repeats (TTGGG)_n [6]. Furthermore, together with her student Carol Greider, they discovered an enzymatic activity in extracts of *Tetrahymena thermophila* and demonstrated the synthesis and elongation of telomeres. Blackburn and Greider termed the activity as telomere terminal transferase, which is known as telomerase [7]. In 1988, the repetitive tandem sequence (TTAGGG)_n was characterized in humans, and it was found to be conserved in all mammals [8].

1.1 Structure and function of telomeres

Telomeric DNA at the end of chromosomes is typically composed of several kilobases of double-stranded repetitive sequence followed by a single-stranded G-rich overhang at the 3' end (Figure 1.1) [9].

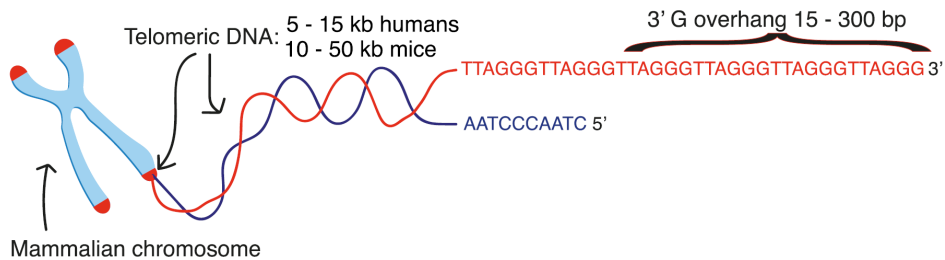


Figure 1.1: Mammalian telomeres. The scheme of a mammalian chromosome highlights telomeres with respect to their key features. The length of telomeric DNA in humans ranges from 5 to 15 kb, and the single-stranded G-rich overhang is up to 300 bp long [10].

The mammalian telomeric sequence consists of (TTAGGG)_n repeats. On the contrary, most plants possess *Arabidopsis thaliana* type tandemly repeated blocks of the sequence (TTTAGGG)_n [8, 11]. Recently, exceptions have been found in plant species, where mammalian-type telomeric repeats are present in the Asparagales order [12]. On the other hand, the *Cestrum elegans* or *Allium* species contain an unusual telomeric sequence motif of (TTTTTTAGGG)_n or (CTCG-GTTATGGG)_n, respectively [13, 14]. The telomeric sequence and the number of repeats are highly conserved in many eukaryotes, whereas in yeast, the sequence and its length are variables [15, 16]. A special case represents *Drosophila melanogaster*, where the terminal tandem array is very different from the canonical telomeric DNA sequence. Telomeres in *Drosophila* use arrays of retrotransposons

to maintain chromosome length, while the vast majority of eukaryotes depend on telomerase-generated tandem repeats [17, 18].

In many animal and plant species, telomeric repeats are located not only at the telomeres but also within the chromosomes as a short tandem array called interstitial telomeric repeats (ITRs). The location of ITRs is often in centromeric regions, and they are likely evolutionary relics derived from chromosomal rearrangements and DNA repair [19, 20].

Telomeres are associated with specialized proteins that shape telomeric DNA [21]. The single-stranded G-rich overhang can invade the double-stranded telomeric part to form a lariat structure called a telomeric loop (T-loop). At the end of the T-loop, a more complex structure called displacement loop (D-loop) is present, where the single-stranded telomeric overhang displaces the G-rich strand of double-stranded DNA and base pairs with an equivalent portion of C-rich strand (Figure 1.2) [22, 23].

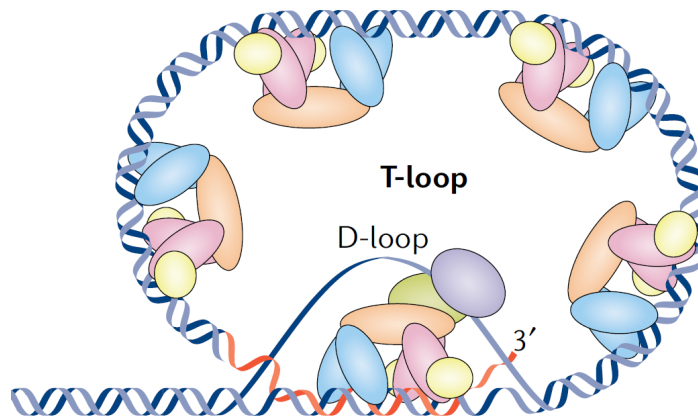


Figure 1.2: Telomeric structure at chromosome ends. G-rich overhang invades the double-stranded telomeric DNA, generating a telomeric (T-loop) and displacement loop (D-loop). In mammals, shelterin complexes regulate the T-loop formation and restrict telomerase access to the single-stranded part of telomeric DNA [23].

The T-loop structure is an essential part of telomeres for its protective role of 3' tail against unwanted DNA damage response (DDR) machinery. Therefore, the single-stranded overhang is not recognized as a DNA double-stranded break and is not subsequently processed by DDR pathways. The T-loop structure is stabilized and regulated in mammals by a protein complex called shelterin

(Figure 1.2) [21, 24, 25].

Due to the guanine-rich tracks, a single-stranded telomeric overhang allows the formation of G-quadruplex (GQ) structures. GQs are nucleic acid structures generated by four single-stranded telomeric repeats [26, 27]. The conformation of GQs involves non-Watson-Crick base pairing. The four guanine nucleotides are paired by Hoogsteen base pairing and stabilized at the center by a monovalent cation (sodium or potassium), creating a G-quartet (Figure 1.3) [28]. GQs affect the replication of telomeres and prevent DNA unwinding, leading to replication fork stalling [29, 30]. Recently, GQs have been shown to modulate telomerase catalysis by increasing the dissociation rate of extended product DNA from telomerase [31, 32].

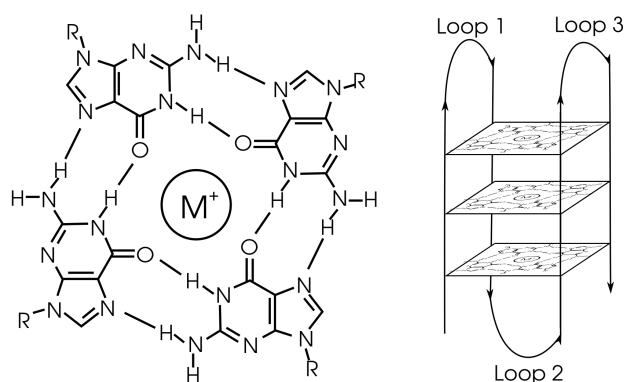


Figure 1.3: The formation of a DNA G-quadruplex (GQ). A G-quartet formed by four guanine nucleotides paired by Hoogsteen base pairing and stabilized at the center by a monovalent cation M^+ (left image), resulting in anti-parallel uni-molecular GQ (right image).

1.2 Replication of telomeres

Every time a cell divides, the genomic DNA is duplicated to be part of each daughter cell. However, due to the inability of conventional DNA polymerases to fully replicate the telomeric sequences at the 3' end, the telomeres progressively shorten with each cell division. Approximately 100 base pairs of telomeric DNA are lost after each round of cell division. The shortening of chromosome ends

limits the lifespan in most cells and ultimately leads to senescence and apoptosis [33–35].

On the other hand, stem and germline cells can divide indefinitely by evading the telomere shortening. Preventing the telomere shortening is also observed in cells during cancer development [36, 37]. More than 85% of cancer cells can circumvent the shortening by the activity of the reverse transcriptase enzyme called telomerase, which elongates telomeric DNA. In up to 15% of tumors, the cells use another mechanism to extend their telomeric DNA termed alternative lengthening of telomeres (ALT), which utilizes homologous recombination (HR) [38, 39].

1.2.1 Telomerase

The ribonucleoprotein (RNP) enzyme complex telomerase catalyzes the synthesis of a new telomere repeat sequence to the 3' end of telomeres. Telomerase can synthesize several telomeric repeats with each binding event, known as telomerase repeat addition processivity [40]. Therefore, providing a telomere maintenance mechanism for germline and somatic stem cells and 85-90% of human cancers [41]. Telomerase comprises a catalytic subunit called telomerase reverse transcriptase (TERT) and the RNA subunit telomerase RNA (TR) [42]. Mutations in TERT that compromise telomerase function results in inherited stem cells dysfunction diseases like dyskeratosis congenita, aplastic anemia, and idiopathic pulmonary fibrosis [42, 43].

The substrate-bound structure of human telomerase holoenzyme shows two flexible RNA-tethered lobes where the catalytic core, including TERT, is flexibly tethered to a H/ACA RNP lobe by TR (Figure 1.4) [44]. The H / ACA lobe contains two RNA hairpins hinged by the H box and flanked by an ACA motif at the 3' end. Each RNA hairpin interacts with heterotetrameric accessory proteins dyskerin, NOP10 (H/ACA ribonucleoprotein complex subunit 3), NHP2 (H/ACA ribonucleoprotein complex subunit 2), GAR1 (H/ACA ribonucleoprotein complex subunit 1), and TCAB1 (telomerase Cajal body protein 1), which drives telomerase to Cajal bodies. Within the catalytic core, TR encircles TERT, which forms a highly organized and restricted structure for protein-RNA interactions. Recently, the structure of DNA and RNA binding interfaces together with the histone H2A-H2B dimer were identified in the catalytic core active site of telomerase [45].

Telomerase is recruited to telomeres during the S-phase of the cell cycle by direct interaction with the telomeric protein TPP1, a part of the shelterin complex. Except for the S-phase of the cell cycle, telomerase is localized to Cajal bodies [46]. Telomerase uses rapid three-dimensional diffusion through

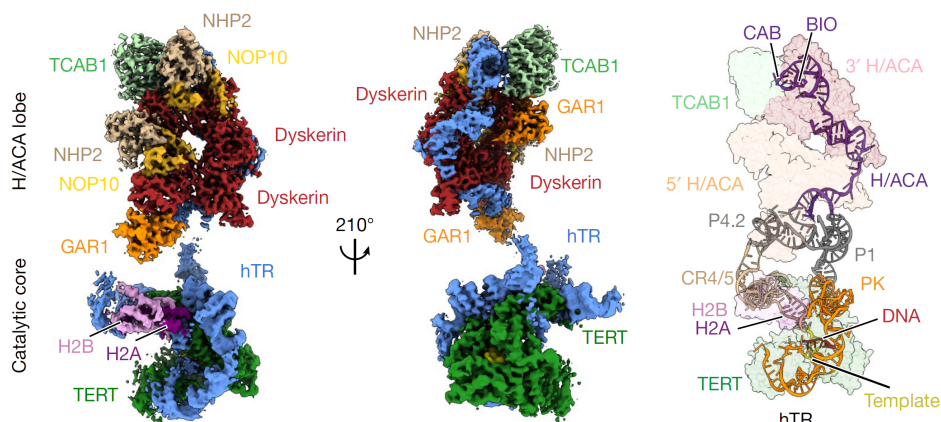


Figure 1.4: The cryo-electron microscopy structure of human telomerase holoenzyme bound to telomeric DNA. The left and middle figures represent front and back views of the Cryo-EM maps of two distinct lobes: catalytic core and H/ACA lobe at 3.8 Å and 3.4 Å, respectively. The figure on the right demonstrates how the DNA substrate is associated with telomerase holoenzyme [45].

the nucleus to search for telomeres, avoiding nucleoli, to dynamically probe each telomere thousands of times. Transient interactions are rarely transformed to a stable formation allowing telomere elongation [47]. The first few nucleotides of the telomeric sequence present a substrate for stable binding at an anchor site within the telomerase. Then the synthesis of new multiple telomeric repeats occurs. After synthesis, the elongated DNA is released from the telomerase in a single step and can be repeatedly bound to the telomerase anchor site together with dynamic folding and unfolding into GQs [32].

1.2.2 Alternative lengthening of telomeres

Cells that lack telomerase activity entirely can still escape the telomere shortening by activating a mechanism termed alternative lengthening of telomeres (ALT). ALT was first identified in yeast and later observed in immortalized human cells without a detectable telomerase activity [48, 49].

Heterogeneous telomere length, together with higher levels of telomere-sister chromatid exchanges (T-SCEs), extrachromosomal telomeric repeats (ECTRs) DNA, and ALT-associated PML (promyelocytic leukemia protein) bodies (APBs) represent essential markers of ALT [50–54].

About 10-15% of human tumors utilize ALT to maintain their telomere length,

rendering the process a potential target for cancer treatment [55]. However, the exact details of the ALT molecular mechanism remain elusive.

ALT is based on a homology-directed DNA repair. Several mechanisms are proposed in which telomeres can use HR to maintain their length [56]. A suggested model exploits unequal T-SCEs, where cells with inherited longer telomeres finally outcompete those with shorter telomeres [57]. Second, the most favored model uses break-induced replication (BIR), a form of HR, where telomeric DNA is synthesized from already existing templates, which could be another telomere or ECTRs DNA [58].

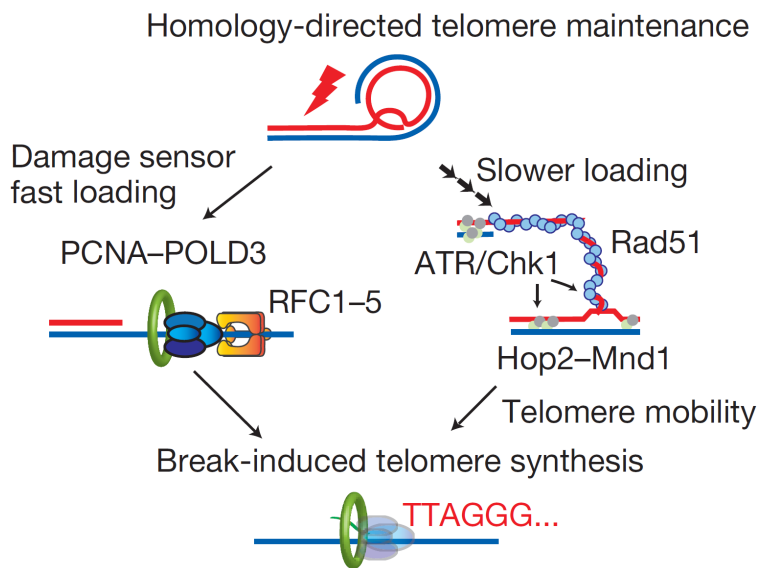


Figure 1.5: Break-induced telomere synthesis in mammalian ALT cells. Double-strand DNA break at telomeres is recognized by RFC (replication factor C), which rapidly recruits the PCNA (proliferating cell nuclear antigen) and DNA polymerase δ . The assembled protein complex synthesizes long tracts of telomeric DNA using a complementary strand as a template. Rad51 and Hop2 loading and long-range homology searches or ATM and ATR are dispensable in break-induced telomere synthesis [59].

Recently, the molecular mechanism of BIR that underlies ALT has been revealed [59]. Dilley *et al.* found that PCNA (proliferating cell nuclear antigen) rapid loading by RFC (replication factor C) initiates double-strand break recognition followed by an assembly of specialized replisome including DNA

polymerase δ . The assembled protein complex can synthesize long tracts of telomeric DNA using a complementary strand as a template (Figure 1.5). The presented mechanism of break-induced telomere synthesis is independent of more slowly occurring long-range homology searches via Rad51 and Hop2 recruitment or ATM (ataxia-telangiectasia mutated) and ATR (Ataxia-telangiectasia and Rad3-related protein). Surprisingly, the Rad51 protein, essential for BIR in yeast, is dispensable in break-induced synthesis in ALT cells [59, 60].

Although the underlying molecular mechanism of ALT in cancer cells has been revealed, there are still many unanswered questions about what induces the ALT mechanism and how it is sustained, or why this method is not active in most cancer cells. Furthermore, the mechanism underpinning the switching or the coexistence of ALT and telomerase in cells remains enigmatic [39, 50].

2 TELOMERE MAINTENANCE

The regulation of telomere length and the protection of chromosome ends are crucial for ensuring genome integrity and stability. The association of specialized protein complexes and their dynamic coordination is required for orchestration such complex processes that shape the telomere structure, guard telomeres against being recognized as a DNA breakage site and mediate telomerase interactions. Shelterin and CTC1–STN1–TEN1 (CST) are two major telomeric protein complexes maintaining the telomeres [23].

2.1 Shelterin complex

In mammals, the telomeric DNA is capped and maintained by a protein complex called shelterin. Shelterin consists of TRF1 (telomeric repeat-binding factor 1), TRF2 (telomeric repeat-binding factor 2), RAP1 (repressor activator protein 1), TIN2 (TERF1-interacting nuclear factor 2), TPP1 (adrenocortical dysplasia protein homolog), and POT1 (protection of telomeres protein 1) [21].

The shelterin proteins TRF1 and TRF2 form homodimers and associate specifically to double-stranded telomeric DNA through their Myb domains [63]. The single-stranded telomeric overhang is bound with a high affinity by POT1, which recognizes the specific telomeric sequence 5'-TTAGGGTTAG-3' through two oligonucleotide/oligosaccharide binding (OB) folds [64, 65]. Additionally, POT1 forms a tight heterodimer with TPP1, promoting POT1 binding to single-stranded telomeric DNA [66, 67]. TPP1 interacts with TIN2 and is directly responsible for telomerase recruitment to telomeres [46, 68]. TIN2 serves as a scaffold that interconnects subunits (TRF1, TRF2, RAP1) attached to double-stranded telomeric DNA to subunits (TPP1, POT1) linked to the single-stranded telomeric overhang [69–71]. RAP1 interacts with TRF2 and improves the binding specificity of TRF2 to double-stranded telomeric DNA [72]. The entire shelterin complex and the interaction map among the associated components are depicted in Figure 2.1.

To date, shelterin presents the only protein complex that binds telomeres

is compromised, leading to genome instability with observed chromosome end fusions [75].

T-loop formation is an important factor in telomere length regulation since it can physically hide the very end of chromosomes from telomerase and DDR sensing [22, 24]. The shelterin proteins TRF1 and mainly TRF2 mediate the T-loop formation, and POT1 stabilizes it [76, 77]. The T-loop size directly depends on telomere length, limiting the total amount of shelterin complexes that can bind to telomeres. Long T-loop with more abundant shelterin complexes has a higher probability for capping the telomeric tail and protecting it against telomerase or DDR. In the case of short T-loop, the situation is exactly the opposite. Due to the lower abundance of shelterin complexes that bind and guard the telomeric tail, telomerase or DDR have a higher probability of accessing the very end of chromosomes [21, 62]. Thus, T-loop formation contributes to telomere maintenance.

2.1.1 TRF1 (telomeric repeat binding factor 1)

TRF1 represents the first described mammalian telomeric protein [78]. It was shown that TRF1 acts as a negative regulator in telomere length due to its ability to inhibit telomerase action through a negative feedback mechanism that stabilizes telomere length. Furthermore, the long-term overexpression of TRF1 protein in telomerase-positive tumor cells leads to gradual and progressive telomere shortening [21, 79].

The majority of telomeric DNA is double-stranded and TRF1, together with TRF2, are the only shelterin factors that directly coat double-stranded telomeric DNA. TRF1 and TRF2 contain a C-terminal DNA-binding Myb domain by which they can specifically bind to the double-stranded telomeric sequence 5'-AGGGTT-3' (Figure 2.1b) [80]. The Myb domains of TRF1 and TRF2 adopt a three-helix structural conformation, which binds the major groove of double-stranded telomeric DNA by its third helix (Figure 2.2) [63, 81]. Furthermore, the mammalian telomeric chromatin architecture consists of nucleosome arrays with 10 bp short linkers between nucleosomes. In this regard, TRF1 can bind to the DNA gyre of nucleosomes, whereas TRF2 interaction is inhibited by nucleosomes, rendering the short DNA linker crucial for the binding of TRF2 [82, 83].

Both TRF1 and TRF2 create homodimers through their telomere repeat factors homology (TRFH) domains, which further enhances the high-affinity binding to double-stranded telomeric DNA [84]. The TRFH domain is linked to the Myb domain by a long flexible linker (approx. 100 and 200 amino acids in TRF1 and TRF2, respectively), allowing the Myb domain of TRF1 and TRF2 to interact with

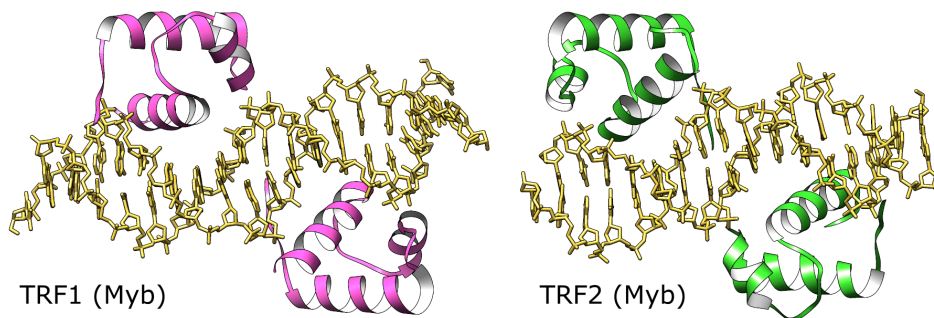


Figure 2.2: The Myb domain of TRF1 and TRF2 bound to double-stranded telomeric DNA. The Myb domain of TRF1 (PDB ID: 1W0T) and TRF2 (PDB ID: 1W0U) is represented as cartoon, and the DNA is rendered in stick representation.

telomeric DNA at various orientations and distances. The flexibility could play a crucial role in binding the chromatin rich in nucleosomes [23, 85]. Despite the low sequence identity (approx. 27%) of TRFH domains of TRF1 and TRF2, their heart-shaped homodimeric structural representations are exceedingly similar. The homodimeric interface is resembled by two stacked helices from each monomer and another helix that helps to stabilize the bundle. The TRFH structures of TRF1 and TRF2 do not heterodimerize due to the differences in charge of interacting side chains and the length of the helix, which would result in a charge and steric hindrance [42, 86]. In particular, the TRFH domain of TRF1 recruits sets of proteins and factors to telomeres, which contain the FxLxP motif (x can be any amino acid). One of the proteins possessing such motif is shelterin unit TIN2.

The significant difference between TRF1 and TRF2 is in their N-terminal domains. While the TRF2 N-terminal part is basic (Arg-rich), TRF1 possesses an acidic (Asp-Glu-rich) N-terminal domain. The contrast in N-terminal domains of TRF1 and TRF2 results in different binding modes of TRF1 and TRF2 to DNA and regulation of the higher-order telomeric DNA structures [21, 23]. The N-terminal acidic domain of TRF1 represses telomeric DNA condensation by suppressing the single-stranded telomeric DNA invasion in T-loop formation [87]. Moreover, the N-terminal acidic domain of TRF1 recruits the tankyrase 1, which can poly(ADP-ribosyl)ate TRF1, leading to releasing TRF1 from telomeres and promoting telomere elongation [88].

TRF1 plays a crucial role in promoting the replication of telomeres, where TRF1 recruits the BLM (bloom syndrome) helicase responsible for unwinding GQ structures, thereby enabling replication and preventing fork stalling [29, 89].

Additionally, TRF1 deletion leads to ATR pathway activation in the S phase of a cell cycle and resembles a fragile-site on telomeres in metaphase. In contrast, the induction of replication stress or BLM removing displays no DNA damage signal despite the evidence of replication problems. Therefore, TRF1 represses the ATR signaling associated with replication stress. [29, 89].

2.1.2 TRF2 (telomeric repeat binding factor 2)

As stated above, TRF2 presents together with TRF1 the only proteins of the shelterin complex that specifically recognize and coat double-stranded telomeric DNA. Figure 2.1b shows the similarity of TRF2 with TRF1 at the domain level, which is supported by their homodimeric spatial conformation [21].

The highly conserved C-terminal Myb domain is responsible for binding to double-stranded telomeric DNA, and homodimerization of TRF2 enhances the DNA binding features (Figure 2.2). Strikingly, TRF2 has an approximately four-fold lower affinity towards telomeric DNA than its counterpart TRF1, despite the exceedingly high amino acid sequence similarity between their Myb domains [63, 84].

TRF2, like TRF1, utilizes its TRFH domain to recognize and bind other partners containing [Y/F]xLxP motif (x can be any amino acid). A few such examples of recruited proteins by the TRFH domain of TRF2 are Apollo proteins, PNUTS (phosphatase nuclear targeting subunit), and MCPH1 (microcephalin 1) [90]. Another important binding partner is REST (RE1-silencing transcription factor), which acts as a master transcriptional regulator and plays a crucial role in neuronal cell differentiation [91, 92]. From a shelterin perspective, the TRFH domain of TRF2 can bind RAP1 with a low affinity, which contains the binding motif YxLxP within its BRCT (BRCA1 C-terminal) domain [93]. Therefore, TRF2 can be viewed as a protein hub recruiting many essential proteins and factors to telomeres.

Recently, the TRFH domain of TRF2 was proposed to wrap approximately 90 bp of telomeric DNA through several lysine and arginine residues, modifying the topology of the telomeric DNA. In contrast, TRF2 wrapping-deficient mutant mediates relaxation of telomeric DNA and displays telomeres to ATM kinase sensing. Interestingly, protection against fusions caused by NHEJ is not impaired in TRF2 wrapping-deficient mutant due to the recruitment of RAP1 protein. Taken together, TRF2-mediated DNA wrapping was proposed to control telomeric DNA topology and ATM inhibition [94].

The N-terminal part of TRF2 (1-45 amino acids) comprises nine positively charged and conserved arginine residues, rendering the N-terminus as a basic (B)-

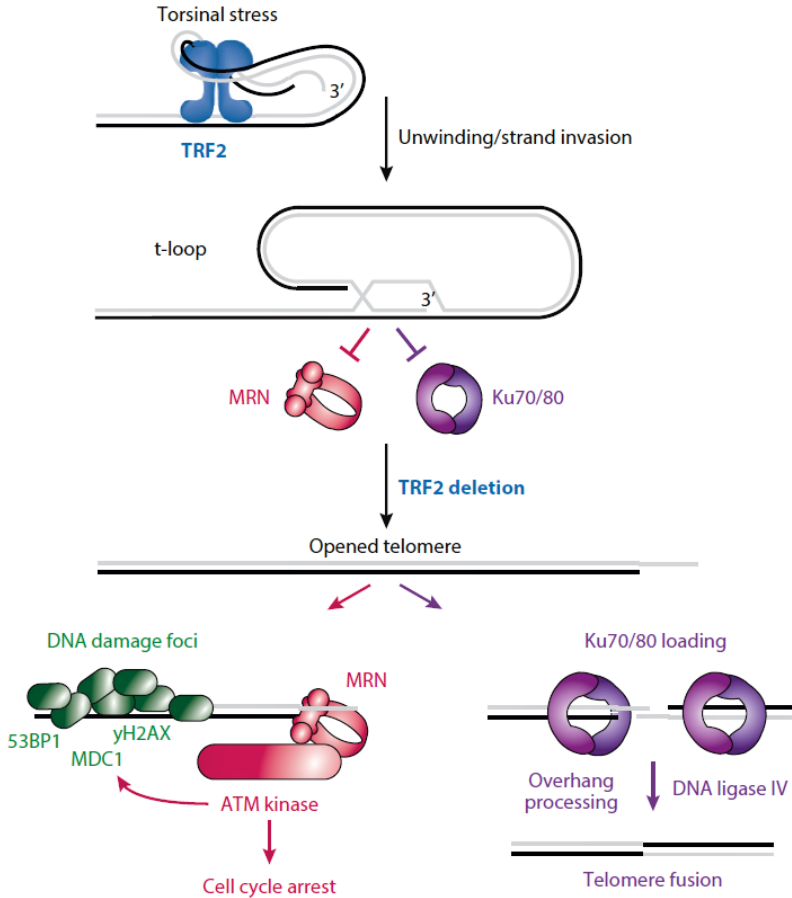


Figure 2.3: TRF2 represses ATM signaling and C-NHEJ at telomeres. TRF2 mediates the T-loop structures, guarding the telomeric tail against MRN complex recruitment leading to ATM activation with further cell cycle arrest and association of other DDR factors on telomeres. Alternatively, TRF2 loss triggers the Ku70/80 loading to open telomeres, leading to C-NHEJ by DNA ligase IV [74]

domain [23, 95]. Given its nature, the B-domain is able to bind the DNA in a non-specific manner. B-domain predominantly binds to Holliday junctions (HJ) or D-loops and protects them from being cleaved by endonucleases, ultimately preventing the deletion of T-loops [96]. TRF2 lacking the B-domain triggers the rapid loss of telomeric DNA due to the T-loop excision by the HR repair mechanism. The B-domain of TRF2 is responsible for blocking the PARP1 (poly(ADP-ribose)

polymerase 1) activation site, which ultimately protects the cleavage of T-loops [97]. Taken together, the B-domain of TRF2 plays an essential role in maintaining the telomere integrity by inducing structural changes to the telomere junction. Therefore, largely contributing to the overall T-loop stability [96, 98]. The critical role of the B-domain is supported by the recent super-resolution microscopy approach revealing that TRF2 is a major factor in telomere chromatin compaction [99]. B-domain of TRF2 is also involved in stabilizing the D-loop formation and reducing the unwinding of telomeric DNA by BLM and RPA (replication protein A) [25].

TRF2 ability to promote the T-loop formation is essential in averting ATM kinase signaling. The model explains that TRF2 prevents loading of DNA double-strand break sensors like MRN (Mre11, Rad50, Nbs1) complex and Ku70/80 heterodimer to the very end of telomeres by hiding the telomeric tail in T-loops (Figure 2.3) [74, 100]. Upon deletion of TRF2, the recruited MRN complex activates the ATM kinase, leading to cell cycle arrest and association of other DDR factors on telomeres. To this extent, Ku70/80 can also bind to the telomeres that lack the lariat T-loop structure and initiate classical NHEJ (C-NHEJ) by DNA ligase IV. The ATM signaling at telomeres is not triggered when other shelterin components are absent [29, 101]. Only TRF2 deletion activates the ATM kinase at telomeres. TIN2 deletion partially activates the ATM kinase at telomeres. However, partial activation supports the impaired stability of TRF2 on telomeres upon TIN2 deletion [69].

The alternative model proposed by Bandaria *et al.*, explaining how TRF2 can avert ATM signaling, is based on the TRF2 mediated compaction of telomeric chromatin. Compacted telomeres by a complex network of shelterin interactions render telomeres inaccessible to DDR sensing. In contrast, decompaction increases the volume of the telomeres and makes the telomeres prone to ATM signaling [99]. Controversially, de Lange and Lingner laboratories shortly after proposed that chromatin decompaction is not required for triggering the DDR at telomeres [102, 103]. Overall, TRF2 plays an essential role in averting ATM signaling at telomeres, and the shelterin-mediated chromatin decompaction is unlikely to be the driven mechanism of repressing the DDR at telomeres.

2.1.3 RAP1 (repressor activator protein 1)

RAP1 presents one of the most evolutionary conserved proteins of the shelterin complex. While in budding and fission yeast, Rap1 can directly bind telomeric DNA. In mammals, the RAP1 is recruited to telomeres through the interaction with TRF2 [21, 104].

Importantly, yeast Rap1 plays a central role in telomere protection against C-NHEJ [105]. However, RAP1 deletion does not directly activate DDR signaling in mammals, and cells lacking RAP1 can survive and are viable [106]. In addition, the involvement of RAP1 in telomere length regulation is still poorly understood [106]. RAP1 can protect critically short telomeres of replicative senescent and tumorous cells upon telomerase inhibition. In cells lacking RAP1, telomere fusions occur when telomerase is inhibited. Moreover, ligase IV is involved in telomere fusions triggered by RAP1 deficiency [107, 108]. Additionally, RAP1 complexed with TRF2, but not TRF2 alone, can inhibit NHEJ at telomeric DNA *in vitro* [109]. Benarroch-Popivker *et al.* recently confirmed that the cooperation of RAP1 with TRF2 wrapping-deficient mutant inhibits telomeric NHEJ [94]. The interaction of RAP1 with TRF2 is also crucial in protecting mammalian telomeres from HDR (homology-directed repair) signaling. Whereas in cells expressing TRF2 mutant unable to bind RAP1, T-SCEs together with chromosome fusions are observed [106, 110].

The interaction of RAP1 with its shelterin partner TRF2 is mediated predominantly through the RCT (RAP1 C-terminal) domain of RAP1 and RBM (RAP1 binding motif) of TRF2 (Figure 2.1b) [23]. Furthermore, RAP1 can also be recruited with a lower affinity by the TRFH domain of TRF2 via its N-terminal BRCT domain [93]. The BRCT domain contains a YxLxP motif specifically recognized by the TRFH domain of TRF2. The assembly of RAP1-TRF2 prevents the unspecific binding of proteins containing the FxLxP motif to TRF2. However, factors that possess the YxLxP motif are not discriminated from binding to TRF2 [93]. Therefore, RAP1 influences the conformation and binding capacity of TRF2 as a protein hub. Furthermore, RAP1 improves the stability and binding specificity of TRF2 towards double-stranded telomeric DNA and DNA junctions [72].

RAP1 is also involved in the NF- κ B signaling pathway in the cytoplasm, which is one of the main non-telomeric roles of RAP1 [111, 112].

2.1.4 TIN2 (TERF1-interacting nuclear factor 2)

As mentioned before, TIN2 is a central component of the shelterin complex, which interacts simultaneously with the factors TRF1 and TRF2 that are responsible for direct binding to double-stranded telomeric DNA. TIN2 also binds to the TPP1-POT1 heterodimer through direct interaction with TPP1 subunit, rendering TIN2 a crucial factor linking the single- and double-stranded telomeric DNA binding units within shelterin [69–71].

Given its bridging features within the complex, TIN2 interactions play a critical role in the end protection function of shelterin. Thus, alternations in TIN2 function

impair telomere maintenance. Chromosomal aberrations and DNA damage are observed in overexpression or knockdown of the TIN2 gene [113]. Significantly, mutations in the TIN2 gene are associated with dyskeratosis congenita (DC), which presents a severe telomeropathy syndrome characterized by bone marrow failure, cancer predisposition, and pulmonary fibrosis. Extremely short telomeres and a more severe form of DC are connected to TIN2 mutations [114, 115]. Moreover, extremely short telomeres in patients with DC-associated mutations in TIN2 occur within a single generation, displaying the change in telomere length as rapid rather than a progressive event [115]. Strikingly, all DC-associated mutations in TIN2 lie in a central region called DC cluster (residues 269 to 298), which partially overlap with a TBM domain responsible for TRF1 (or TRF2) binding. However, no direct effect of DC-associated mutations was observed on binding with shelterin partners, suggesting the impact on interactions with other factors [116–118].

TIN2 is present in two main isoforms in cells, TIN2S (short) and TIN2L (long), which have a unique role in telomere maintenance. It was shown that only TIN2L could be phosphorylated, which enhances the binding to TRF2. In contrast, TRF1 interacts more with TIN2S than with TIN2L. Overexpression of TIN2L promotes telomere elongation, while overexpression of TIN2S or even phosphodead TIN2L mutant does not [118].

TIN2 contributes to telomere length regulation by stabilizing the TRF1 association with telomeres. Therefore, TIN2 represses the releasing of TRF1 from telomeres caused by poly(ADP-ribosyl)ation of TRF1 [119]. Moreover, TIN2 protects TRF1 from ubiquitination [120]. However, the lowered amount of TRF1 on telomeres due to TIN2 silencing is likely sufficient for the proper telomere replication [69].

In the case of TRF2 binding, TIN2 largely contributes to the TRF2 driven ATM repression. It was shown that TIN2 deletion triggers the ATM signaling pathway with observed chromosome-type telomere fusions, which is a typical phenotype for TRF2 loss. Strikingly, no other shelterin partner can enhance ATM signaling repression by TRF2 as TIN2 does [69, 121].

Importantly, TIN2 tethering to TPP1-POT1 heterodimer is required for repressing ATR signaling by POT1. The TPP1-POT1 interaction with the shelterin core protein TIN2 stabilizes POT1 on single-stranded telomeric DNA and allows POT1 to outcompete RPA, despite having the same affinity for single-stranded telomeric overhang [69, 74]. Moreover, TIN2 binding to TPP1 helps to repress both C-NHEJ and alternative NHEJ (A-NHEJ)-mediated chromatid and sister telomere fusions [70].

Notably, the deletion of TIN2 protein showed the same phenotype as cohesin

subunit SA1-depleted cells, where the maintenance of cohesion between sister telomeres after DNA replication in the S phase was impaired [122]. The phenotype similarity suggests that TIN2 contributes to chromatin architecture and genome maintenance.

2.1.5 TPP1 (adrenocortical dysplasia protein homolog)

Shelterin component TPP1 represents an essential factor within the complex that helps to interconnect the subunits between single- and double-stranded telomeric DNA by simultaneous binding to TIN2 and POT1 [21, 74]. The crucial role of TPP1 involves telomerase recruitment to telomeres, which is achieved by direct interaction with the TEN (telomerase essential N-terminal) domain of TERT [123]. Additionally, TPP1 promotes telomerase repeat addition processivity and facilitates telomere elongation in telomerase-positive cells [46, 124, 125].

The specific region that mediates the telomerase interaction has been identified within the OB domain of TPP1 (Figure 2.1b) [46]. The surface of TPP1 OB-fold contains seven conserved residues collectively termed as a TEL (TPP1 glutamate (E) and leucine (L)-rich) patch that is crucial for binding to TEN domain of telomerase (Figure 2.4a). Three critical glutamate residues, E168, E169, and E171, create a flexible loop within the interaction surface known as a TEL patch knuckle that protrudes from the five-stranded β -barrel structure of OB-fold and terminates in a short α -helix (Figure 2.4b) [46, 126, 127]. The deletion of single lysine residue Δ K170 disrupts the TEL patch knuckle structural conformation by displacing glutamate residues E168 and E169, leading to loss of TPP1 telomerase recruitment function [128]. Furthermore, TPP1 Δ K170 is associated with telomere shortening and telomeropathies, where patients suffer from a severe form of DC [128, 129]. A separate region in the N-terminus of the OB domain (termed NOB) of TPP1 was recently identified. Largely hydrophobic NOB region, together with the acidic pocket of the TEL patch, is essential for telomerase recruitment to telomeres and telomerase repeat addition processivity [61].

Taken together, two surfaces of TPP1 that are critical for telomerase function at telomeres have been characterized. However, the interaction site on telomerase that directly interacts with TPP1 is not well defined. Only two residues, K78 and R132, within the TEN domain of human TERT, directly involved in salt bridging interaction with TEL patch of TPP1, are mapped [123]. Additionally, the CTE (carboxy-terminal extension) domain of TERT has been suggested to bind the NOB region of TPP1 (Figure 2.1b) [61, 62].

The nuclear localization of TPP1 is mediated by binding to TIN2 protein in the cytoplasm. Furthermore, TPP1 contains two functional nuclear export signals

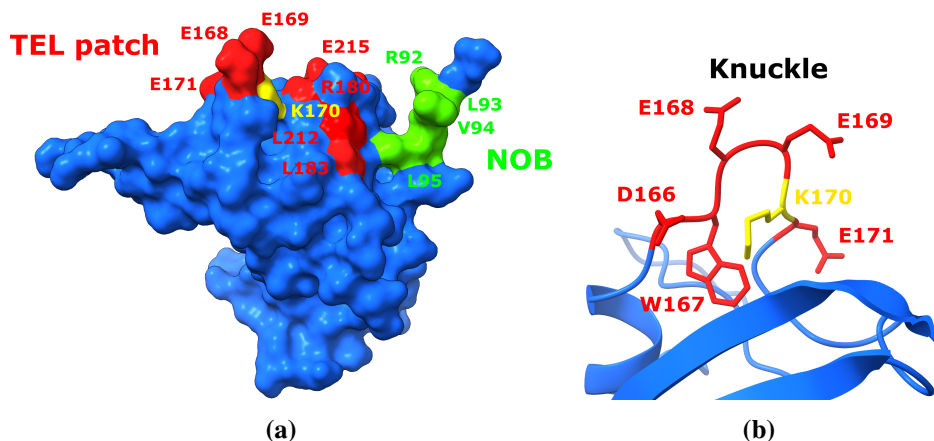


Figure 2.4: The interaction surface of TPP1 OB-fold. (a) The crystal structure (PDB ID: 2I46) of the TPP1 OB-fold (blue) with highlighted TEL patch (red) and NOB region (green). K170 residue, associated with dyskeratosis congenita (DC), is depicted in yellow. (b) The representation of TEL patch knuckle (PDB ID: 2I46) with critical amino acids E168, E169, and E171 that are directly responsible for telomerase binding [128].

proposed to control the amount of TPP1 and POT1 in the nucleus. Nevertheless, the exact mechanism of how TPP1 cooperates with POT1 and TIN2 to regulate the number of shelterin proteins in the nucleus by dynamic trafficking to the cytoplasm is yet to be addressed [130].

The interaction of TPP1 with POT1 dramatically promotes POT1 binding to single-stranded telomeric overhang [67, 124]. Moreover, TPP1 also enhances the POT1 telomeric protective function against DDR by contributing to repressing ATR signaling [69, 131, 132].

TPP1 contains multiple sites that are phosphorylated during the cell cycle progression. In particular, phosphorylation of Ser111 within the OB-fold during the S/G2/M phase of the cell cycle promotes telomerase recruitment to telomeres [133]. The long and flexible Ser/Thr-rich region of TPP1 that connects the POT1-binding domain with the TIN2-binding domain contains seven highly conserved Ser/Thr phosphorylation motifs (Figure 2.1b). The function of conserved motifs is still unclear. Although possible post-translational modification (PTM) states of the Ser/Thr-rich region could regulate shelterin organization during the cell cycle and modulate the behavior of TPP1 [23, 133].

2.1.6 POT1 (protection of telomeres 1)

The telomeric protein POT1 presents the most conserved component of the shelterin complex. POT1 was identified together in humans and fission yeast by homology search for telomeric single-stranded DNA binding proteins in ciliates [21, 134]. On the contrary, two closely related proteins, POT1a and POT1b, originated by gene duplication, are part of the shelterin complex in rodents [135].

POT1 guards the single-stranded telomeric overhang by specific nanomolar affinity binding to telomeric sequence through two N-terminal OB-folds (Figure 2.1b) [64, 136]. Figure 2.5 demonstrates how the telomeric single-stranded DNA is kinked between OB1- and OB2-folds, while OB1-fold interacts with the first six bases of the 5'-TTAGGGTTAG-3' sequence [136]. Notably, POT1 binds the telomeric overhang in a 3' to 5' direction [137].

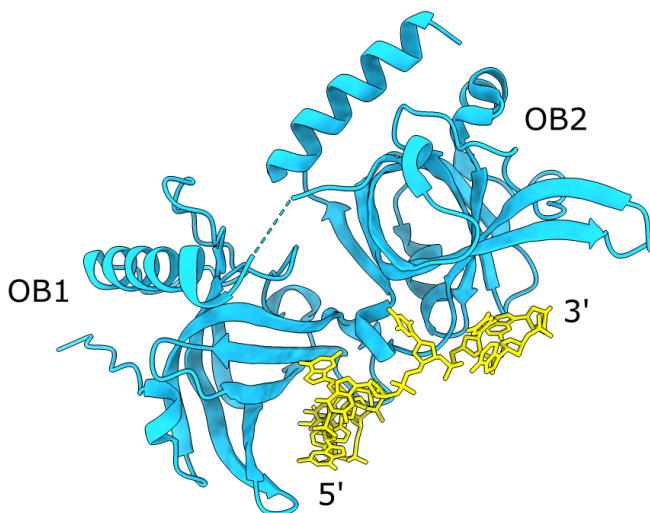


Figure 2.5: The DNA binding domain of POT1 bound to single-stranded telomeric DNA. The OB1 and OB2 domains of POT1 are represented as cartoons, and the DNA sequence 5'-TTAGGGTTAG-3' is rendered in stick representation (PDB ID: 1XJV).

Furthermore, POT1 associates with TPP1, which promotes POT1 binding to telomeric overhang and increases the POT1 preference to bind DNA over RNA [67, 138]. According to structural data, the TPP1 binding domain of POT1 lies in the C-terminal part of POT1 and contains one OB-fold and Holliday junction resolvase-like domain. Importantly, both domains are essential for binding

to TPP1, and the overall POT1-TPP1 structural interface resembles an elongated V-shaped conformation [139]. It was shown that the N-terminal part of POT1 containing DNA binding domain and the POT1-TPP1 heterodimers could completely coat long telomeric single-stranded DNA substrates *in vitro*. Additionally, the telomeric overhang coated with POT1-TPP1 heterodimers forms compact and potentially ordered structures [67].

POT1 plays a crucial role in telomere length regulation in humans. A POT1 mutant lacking the DNA binding domain diminished the TRF1 mediated control of telomere length, which resulted in rapid telomere elongation [140]. Another study demonstrated that POT1 could strongly inhibit telomerase activity when it binds to the second last telomeric repeat array, leaving less than a minimum length of telomeric substrate accessible for telomerase [136]. As a result, the negative telomere length regulation by POT1 affects the substrate access to telomerase [141]. In contrast, POT1 can also positively regulate telomere length and facilitate telomere elongation by telomerase [142].

Additional activity of POT1 to promote the telomere elongation is the ability to disrupt GQs upon binding to single-stranded telomeric overhang [143]. Complexation with the TPP1 partner displays a highly dynamic sliding movement along single-stranded telomeric DNA, further contributing to the unfolding and refolding of GQs [137].

POT1 binding to single-stranded telomeric overhang blocks the RPA loading at telomeres. Thus, POT1 prevents the activation of the ATR signaling pathway. The ATR repression is strengthened upon POT1 binding to TPP1 and TIN2 proteins within the shelterin complex [74, 101]. Moreover, POT1 mutations, which naturally occur in the TPP1 binding domain at C-terminus, trigger the DDR pathway resulting in genomic instability and cancer [139]. Overall, POT1 fulfills critical telomere length regulation and chromosome end protection functions.

2.2 Shelterin assembly and dynamics

Shelterin represents a highly dynamic complex formed through interactions between its subunits and telomeric DNA. Despite a considerable number of insights revealing how the shelterin is formed, the detailed whole complex assembly mechanism still presents one of the major questions in the telomere biology field. Moreover, studies describing the mutual binding relationships within shelterin often result from binary interaction analysis limited to their interaction domains [23, 62].

From the shelterin scheme (Figure 2.1a) is evident that the TIN2 subunit acts as a bridging factor, which interconnects shelterin subunits attached to double-

stranded DNA and subunits attached to the single-stranded telomeric overhang. The interaction of TIN2 with TPP1 was proposed to be essential for mediating higher-order shelterin formation [68]. Specifically, TPP1 enhances the stability of the TRF1-TIN2-TRF2 subcomplex [68]. Recently, the simultaneous cooperation of TIN2 with TPP1 and TRF2 has been described by Hu *et al.* [70]. The structural study showed how the long α -helix that lies in the N-terminal part of TIN2 acts like a seesaw, which binds TPP1 to one end and TRF2 to its other. Thus, the simultaneous interaction at several binding sites further enhances the binding capacity of TIN2 [70].

The recent attempt to define the human shelterin complex *in vitro* is based on the reconstitution of human shelterin lacking TRF1 from insect cells [71]. The study of Lim *et al.* revealed the POT1:TPP1:TIN2:TRF2:RAP1 stoichiometry of 1:1:1:2:2 and showed that a complex containing both TRF2 and POT1 has the strongest affinity to bind telomeric DNA junctions. However, no structural insights on how the purified shelterin complex assembles were described [71].

Newly, the reconstitution of human shelterin subcomplexes and the entire six-subunit complex were reported [144]. The study employed single-particle negative-stain electron microscopy to characterize the recombinantly produced human shelterin complex. The obtained results highlight the extreme structural heterogeneity and dynamics of shelterin units and confirm that the whole shelterin can assemble without telomeric DNA substrate [145]. Zinder *et al.* observed the fully dimeric stoichiometry of the shelterin complex. Furthermore, the data confirmed the high flexibility of the hinge region interconnecting the N- and C-terminal part of POT1, which is transferred to the TPP1 OB-fold domain and TIN2 TRFH domain [146]. Unfortunately, extreme heterogeneity prevents the structural characterization of the whole shelterin complex due to the numerous possible spatial conformations.

While the newest data shed important light on the architecture of human shelterin, the high-resolution structure is still intractable. The characteristic flexibility and dynamic properties of shelterin may be a crucial advantage in fulfilling many functions at once. For example, the ability of shelterin to bind telomeric DNA at different conformations and simultaneously accommodate various factors involved in the telomere maintenance [23, 144].

If the elucidation of human shelterin architecture *in vitro* has been so far highly challenging, studying the shelterin formation and dynamics *in vivo* presents an even more difficult task. Here, only a handful of research articles were published, which broadened the knowledge of shelterin assembly mechanism in living cells.

The rough estimation of each shelterin component in cells has been carried out

by de Lange laboratory. The telomeric double-stranded binding factors TRF1 and TRF2 were sufficiently abundant for binding all telomeric DNA in cells with short telomeres. On the other hand, TPP1-POT1 was present only in 50-100 copies per telomere. However, this should be enough to cover single-stranded telomeric overhangs. RAP1 showed 1:1 stoichiometry with TRF2. TIN2 was in 10 fold excess compared to TPP1-POT1, and the abundance of TIN2 allowed binding of each TRF1 and TRF2 molecule [111].

In addition, the uneven stoichiometry of chromatin-bound shelterin has been proposed, where the most abundant subcomplex consists of TRF2 homodimer, two RAP1, and one TIN2 molecules. The second most abundant shelterin subcomplex comprises the same units with addition of the TRF1 homodimer. Strikingly, TPP1-POT1 heterodimers are bound to only about 10% of these subcomplexes, addressing that the six-subunit complexes represent approximately 5% of all shelterin formations. [23, 111]. The uneven stoichiometry raises the question of why shelterin is in cells most of the time disassembled, but mainly if it needs to be fully assembled for an optimal function as a primary telomere guardian.

2.3 CST (CTC1–STN1–TEN1) complex

The CST complex complements shelterin, and together they play an essential role in the overall telomere maintenance. Mammalian CST presents a heterotrimeric complex identified based on homology search to the yeast CST due to their similarity to RPA complex in terms of single-stranded DNA binding properties through contained OB domains [147, 148].

The mammalian CST is involved in telomere replication. It was shown that the depletion of CTC1 or STN1 subunit results in telomeric overhang extension. The extension occurs because CST serves as a DNA polymerase α -primase (Pol- α) cofactor that mediates the subsequent telomeric C-strand fill-in process and controls the duplication of lagging-strand for the proper synthesis of telomeric DNA [149, 150].

The single-stranded DNA binding properties of CST allow it to compete with shelterin TPP1-POT1 factors for single-stranded telomeric DNA binding. The CST competition with TPP1-POT1 can ultimately stop telomere extension by telomerase since TPP1 serves as a direct factor for telomerase recruitment. Moreover, CST facilitates the C-strand fill-in process involving Pol- α primase. The termination of telomerase activity by CST complex is also supported by *in vivo* study, where CST disease mutation increases telomerase recruitment to telomeres [151, 152].

Recently, the cryo-electron microscopy structure of human CST complex

3 AIMS OF THE THESIS

The main focus of the thesis is to describe the shelterin assembly, which has a critical role in telomere maintenance. The particular aims include establishing a single-molecule technique based on fluorescence cross-correlation spectroscopy (FCCS) and utilizing it to elucidate the complexation of TRF1, TRF2, and TIN2, representing the core of the shelterin complex. Second, characterizing the changes in TIN2 binding capacity upon TPP1 addition and assessing the effect of TPP1 on shelterin core assembly. Furthermore, complement the single-molecule results of shelterin core assembly with ensemble measurements and define the DNA binding properties of the reconstituted complex.

- Describing the shelterin core formation at the single-molecule level.
- Characterization of how TPP1 affects the shelterin core assembly.
- Revealing the molecular mechanism of shelterin core formation and defining the potential weakest point in telomere maintenance.

4 METHODS

A single-molecule approach that utilizes fluorescence cross-correlation spectroscopy (FCCS) was applied to assess shelterin core assembly in solution. At the same time, fluorescence-based biophysical methods were complemented to quantify interactions within shelterin. Here, the essential methods are briefly described, with a primary focus on principles of used methodologies and techniques.

4.1 Fluorescence correlation spectroscopy

The majority of fluorescence-based techniques describe the biomolecular properties in an ensemble. The analyzed parameters then represent an average value of the measured population of molecules. Fluorescence correlation spectroscopy (FCS) can overcome the limitation of ensemble methods by lowering the concentration of analyzed molecules and minimizing the detection volume. Thus, FCS enables monitoring each molecule individually and obtaining the studied parameters at single-molecule resolution. FCS provides information about the kinetic and thermodynamic properties of fluorescently labeled molecules diffusing in an aqueous buffer solution. FCS directly measures the spontaneous intensity fluctuations caused by individual fluorophores passing through the detection volume [155–157].

A confocal microscope is generally used to perform the FCS experiments. The excitation continuous-wave laser light is focused by a high numerical aperture objective to a diffraction-limited spot. Confocal pinhole further limits the detection volume in the axial direction by passing the emission light only from a focal region. Combining the confocal setup, which minimizes the detection volume below one femtoliter, with an extremely low sample concentration (nanomolar to picomolar), guarantees the single-molecule precision [155, 157].

The statistical approach must be applied to quantitatively analyze the temporal fluorescence intensity fluctuations of studied molecules. Autocorrelation of a signal representing the measured intensity fluctuations provides information

about important biochemical parameters of underlying dynamic processes. The autocorrelation function $G(\tau)$ is defined as correlation of a given signal with itself shifted by a lag time τ :

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2}, \quad (4.1)$$

where $\delta I(t)$ represents the fluorescence intensity deviation from the average fluorescence intensity $\langle I(t) \rangle$. The autocorrelation amplitude $G(0)$ at $\tau = 0$ is inversely proportional to the average number of molecules N in the detection volume. Therefore, if the detection volume size is known, the mean concentration can be determined. Due to the extremely low sample concentration, the number of molecules within the detection volume obeys a Poisson probability distribution. A typical autocorrelation curve (Figure 4.1) has a characteristic decay time from which the diffusion coefficient of monitored molecules can be derived, assuming that the detection volume size is known [157–159].

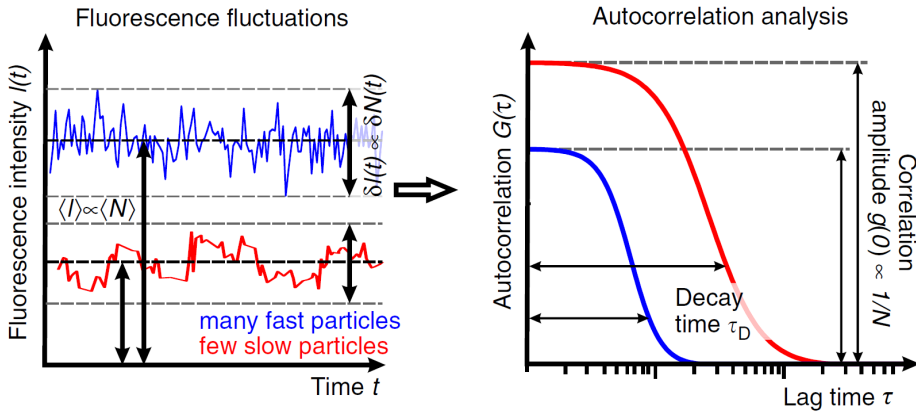


Figure 4.1: Autocorrelation analysis in FCS. Fluorescently labeled molecules are monitored as they diffuse through the detection volume of a confocal microscope. The autocorrelation function $G(\tau)$ describes the spontaneous fluorescence intensity fluctuations. At $\tau = 0$, the amplitude $G(0)$ is inversely proportional to the average number of molecules N in the detection volume [159].

FCS can also be applied for studying molecular interactions. The binding of fluorescently labeled molecules is then monitored from the change in diffusion time of the fluorophore. To detect the changes in diffusion time, the bound complex has to differ in molecular mass at least by a factor of 4, which makes

this approach insensitive for studying molecular interactions of similar molecular masses. To overcome the limitation, fluorescence cross-correlation spectroscopy (FCCS) has been developed to quantitatively measure the molecular interactions of two or more differently fluorescently labeled molecules [160].

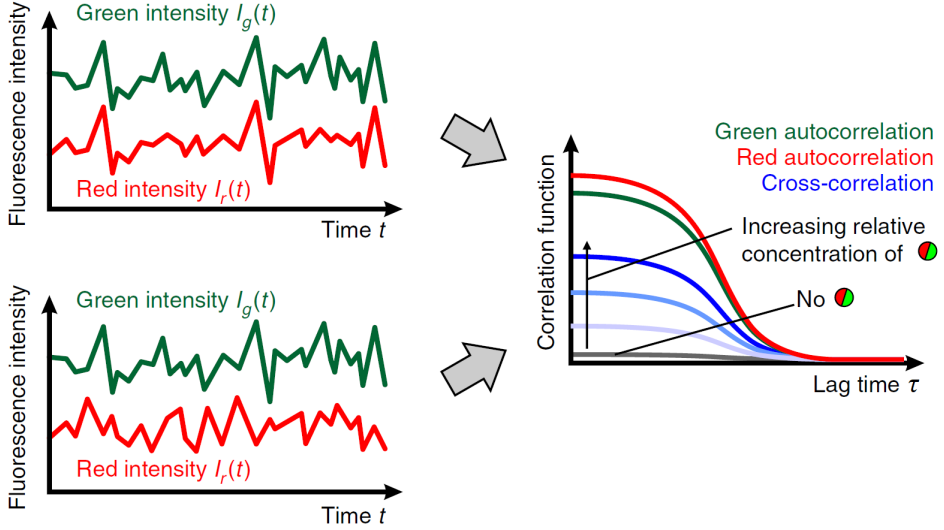


Figure 4.2: The general concept of FCCS. Green and red fluorescently labeled molecules diffuse through the illuminated detection volume, and their intensity fluctuations are monitored. If green and red molecules interact with each other, their fluorescence intensity fluctuations have similar patterns; hence the amplitude of the cross-correlation function increases accordingly [159].

For two-color FCCS, the two different fluorescence signals are monitored simultaneously and further analyzed how their intensity fluctuations correlate to each other (Figure 4.2). The cross-correlation function $G_{gr}(\tau)$ of a two-color system with one green-labeled particle g and the second red-labeled particle r , is described as follows:

$$G_{gr}(\tau) = \frac{\langle \delta I_g(t) \cdot \delta I_r(t + \tau) \rangle}{\langle I_g(t) \rangle \cdot \langle I_r(t) \rangle}, \quad (4.2)$$

where $\delta I_g(t)$ and $\delta I_r(t)$ represent the individual fluctuations in fluorescence intensity for each labeled particle (green and red) from their average intensity $\langle I_g(t) \rangle$ and $\langle I_r(t) \rangle$, respectively. Lag time is denoted by τ , which can be interpreted as a period in which the two analyzed molecules stay in detection volume together.

At $\tau = 0$, the cross-correlation amplitude $G_{gr}(0)$ is inversely proportional to the average number of bound species, assuming the ideal conditions of no fluorescence resonance energy transfer and spectral cross-talk of used fluorophores [157, 159, 161].

4.2 Fluorescence anisotropy

One of the most broadly used biophysical techniques for binding affinity analysis is fluorescence anisotropy (FA). FA directly measures the rotational mobility of molecules with respect to the time between the absorption and emission events. The ground-state fluorophores are all randomly oriented in a homogeneous solution. The existence of transition moments for absorption and emission that lie along specific directions within the fluorophore structure is the origin of anisotropy [162].

To perform an interaction measurement of molecules of interest, one is supposed to be fluorescently labeled. If the sub-population of fluorophores are selectively excited with vertically polarized light, the emission retains that polarization to some degree based on the rotational mobility of analyzed molecules. In other words, if the fluorescently labeled molecules tend to rotate very fast, the emission light will retain less amount of polarization and vice versa [162–165].

FA r is defined as

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}, \quad (4.3)$$

where $I_{\parallel}$ is the observed intensity when the emission polarizer is oriented parallel to the direction of polarized excitation. In the same way, $I_{\perp}$ is the intensity of the perpendicularly oriented emission polarizer to the excitation. According to equation 4.3, FA is independent of the fluorophore concentration, which is an important property of anisotropy [162, 166].

A concept of FA assay, depicted in Figure 4.3, is generally used to measure and subsequently quantify the binding of a small fluorescently labeled molecule (typically an oligonucleotide, peptide, or protein) to a protein with a higher molecular weight. The fluorophore attached to a small molecule is excited with polarized light. Due to the rapid Brownian motion, the fluorophore reorients during its excited state's lifetime; hence, the emitted light will be highly depolarized (low anisotropy). On the other hand, if the labeled molecule is bound to a protein with a higher molecular weight, the complex tumbles much slower, which results in a much smaller amount of fluorophore reorientation; thus, the emitted light retains its polarization (high anisotropy) [166, 167].

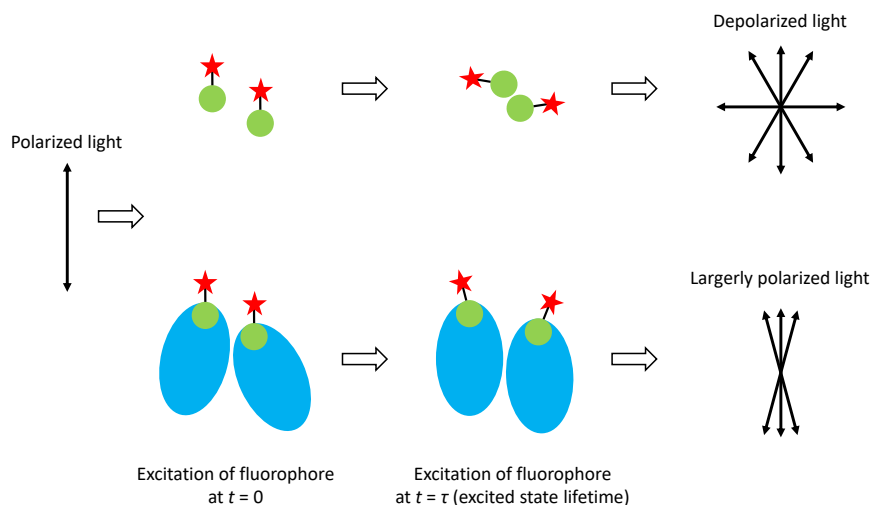


Figure 4.3: The general concept of fluorescence anisotropy. If the polarized light excites a small biomolecule (green circle) with a fluorescent label attached (red star), the molecule reorients significantly due to the rotational movement. The fast random reorientation results in a largely depolarized emitted light, hence low anisotropy. When a higher molecular weight protein (blue ellipse) is bound to the fluorescently labeled small molecule, the resulting complex rotates much slower, and the emission retains its polarization, hence high anisotropy.

4.3 Microscale thermophoresis

Fluorescence-based methods have become a fundamental tool used in biophysical studies for the quantitative determination of biomolecular interactions. Microscale thermophoresis (MST) is a robust and powerful technique that belongs to this category. MST is highly sensitive to various molecular properties, making it valuable for quantitative analysis of protein interactions independent of the size, charge, hydration shell, or conformation of studied biomolecules. The technique is based on thermophoresis, the directed diffusional flux of molecules in a temperature gradient [168, 169].

MST monitors the directed movement of fluorescently labeled molecules through a microscopic temperature gradient induced by an infrared (IR) laser. The movement of molecules is described with a linear drift response. The molecular flow j is proportional to the temperature gradient $gradT$ with the thermal diffusion

coefficient D_T , where c refers to a molecule concentration:

$$j = cD_T \text{grad}T. \quad (4.4)$$

In a steady-state, the drift is compensated by mass diffusion [169, 170].

Due to the binding-induced changes in molecular properties (charge, size, and solvation energy), the thermophoresis of fluorescently labeled molecules differs significantly from the thermophoresis when a protein is bound to a studied fluorescently labeled molecule. Therefore, MST can detect binding even if the change in molecular size is negligible. MST can be performed in almost any buffer, even in a crowded solution like cell lysate, which can quantify the molecular interactions in a near-native state. Finally, the sample consumption is also very low. Only a few microliters are generally sufficient to perform a precise measurement. These mentioned advantages circumvent the limitations of other commonly used fluorescence-based methods [168, 171].

For molecular interaction analysis, the affinity is quantified by the change in normalized fluorescence F_{norm} , which is defined as a function of the concentration of binding partner:

$$F_{norm} = (1 - x)F_{norm}(\text{unbound}) + xF_{norm}(\text{bound}), \quad (4.5)$$

where $F_{norm}(\text{unbound})$ and $F_{norm}(\text{bound})$ represent the normalized fluorescence of unbound labeled molecules and formed complex, respectively. The fraction of labeled molecules bound to their binding partner is denoted by x [170].

The general experimental setup consists of an IR laser focused on the sample and a coupled fluorescence excitation and detection. For a typical interaction analysis, serial dilution of up to 16 samples is used. MST is measured in a thin glass capillary where the fluorescence of the labeled molecules is monitored before, during, and after the IR laser locally induced heating. A typical MST signal for a given capillary represents figure 4.4. Several subsequent processes can be observed during the experimental timeline and dependence on the IR laser heat source. Initially, the fluorescence of the sample at experimental temperature is recorded, followed by a change (decrease) in fluorescence due to the sample heating induced by an IR laser. The change in fluorescence is called MST T-Jump and is observed just after turning on the IR laser and before the molecule's thermophoretic movement occurs. Then, the thermophoresis induces changes in fluorescence due to the thermophoretic motion of molecules. Finally, after turning off the IR laser, an inverse T-Jump is observed caused by sample cooling, followed by a fluorescence recovery driven by mass diffusion of molecules [170, 172].

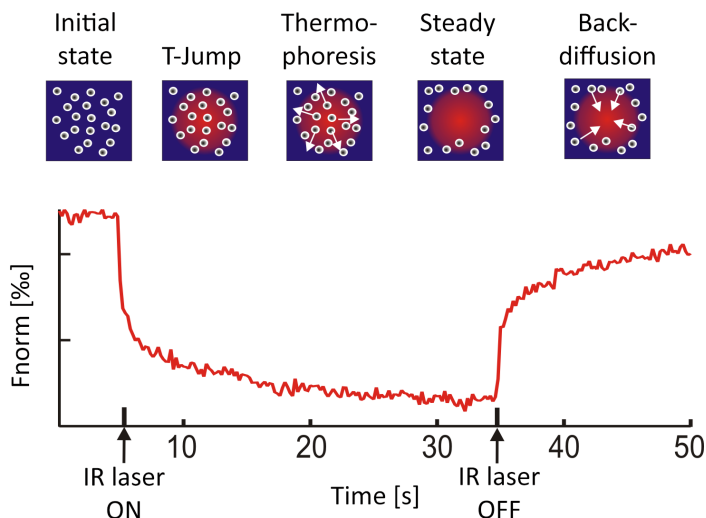


Figure 4.4: Typical MST signal for a given capillary. Firstly, the initial fluorescence is measured at experimental temperature without infrared (IR) laser-induced heating. After turning on the IR laser, a temperature jump (T-Jump) is observed, followed by a molecule movement during a thermophoresis (lasts about 25 s). Inverse T-Jump is observed when the IR laser is turned off, and the fluorescence recovery driven by mass diffusion of molecules occurs [170].

4.4 Size-exclusion chromatography

During the protein purification, size-exclusion chromatography (SEC) became an invaluable separation-based technique that is routinely used as the last step of the purification protocol. SEC separates molecules according to their hydrodynamic radius as they pass through a chromatography column packed with a resin. The stationary phase consists of a fairly defined porous matrix. When a sample is injected and eluted through the column, the molecules can diffuse into the gel differently depending on their size. Small molecules can penetrate the pores and stay in the gel for a more extended period, and consequently, they will elute last. On the other hand, molecules larger than the pores that cannot diffuse into the sorbent particles will be excluded, and they will elute first. A steric hindrance limits access to the pores [173, 174].

The SEC has a broad range of applicability. In particular, SEC is a well-established technique used in sample quality control to analyze the sample heterogeneity and to detect the presence of aggregates. SEC is often used to quantify

protein oligomeric states due to the sufficient resolution to separate different molecular assemblies. Therefore, SEC can be utilized for analyzing the formation of various complexes as it allows size profiling of protein samples due to the mild separation conditions [174, 175].

5 AUTHOR'S PUBLICATIONS AND CONTRIBUTION

Here is a list of first- and co-authored publications included in the doctoral thesis, together with my contribution to each article.

- **Research article 1** (page 87)

Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 When TPP1 Is Absent

T. Janovič, M. Stojaspal, P. Veverka, D. Horáková, C. Hofr
Journal of Molecular Biology (2019), DOI: [10.1016/j.jmb.2019.05.038](https://doi.org/10.1016/j.jmb.2019.05.038)

Contribution:

- I designed the project's main experimental direction.
- I prepared DNA constructs used for protein recombinant expression.
- I participated in the expression and purification of shelterin proteins.
- I fluorescently labeled the purified proteins.
- I performed all of the FCCS experiments and analyzed the FCCS data.
- I carried out the SEC experiments with controls by dynamic light scattering (DLS).
- I measured MST and quantified the binding affinities of studied shelterin proteins.
- I prepared the figures and co-wrote the manuscript.

- **Research article 2** (page 101)

Enhancement of luminescence signal by deuterated water – Practical implications

J. Kučera, O. Peš, T. Janovič, C. Hofr, L. Kubinyiová, J. Tóth, Š. Káňa, P. Tábořský
Sensors and Actuators B: Chemical (2022), DOI: [10.1016/j.snb.2021.131029](https://doi.org/10.1016/j.snb.2021.131029)

Contribution:

- I designed the experimental direction for the fluorescence microscopy section.
- I performed the FCS experiments and analyzed the FCS data.
- I transfected and cultivated a mammalian cell line transiently expressing the fluorescent proteins used for fluorescence lifetime microscopy (FLIM) experiments.
- I conducted the FLIM measurements on a living-cells sample and analyzed the FLIM data.
- I conducted the immunofluorescence (IF) experiment on fixed mammalian cell lines.
- I performed the standard wide-field fluorescence microscopy on samples prepared by IF.
- I summarized the microscopy section in the prepared manuscript.

- **Review article 1** (page 110)

Quantitative Biology of Human Shelterin and Telomerase: Searching for the Weakest Point

P. Veverka, T. Janovič, C. Hofr
International Journal of Molecular Sciences (2019), DOI: [10.3390/ijms20133186](https://doi.org/10.3390/ijms20133186)

Contribution:

- I participated in designing, writing, and editing of the manuscript.
- I wrote the section dedicated to the quantitative description of interactions within shelterin.

- **Review article 2** (page 124)

Transcriptional regulators of human oncoviruses – structural and functional implications for anticancer therapy

I. Nečasová, M. Stojaspal, E. Motyčáková, T. Brom, T. Janovič, C. Hofr
NAR Cancer (2022), DOI: [10.1093/narcan/zcac005](https://doi.org/10.1093/narcan/zcac005)

Contribution:

- I participated in designing, writing, and editing of the manuscript.
- I co-wrote the section dedicated to the Epstein-Barr virus (EBV).
- I wrote the section dedicated to Rta (replication and transcription activator) factor.

6 COMMENTARY ON THE SCIENTIFIC ARTICLES

The molecular mechanism of human shelterin assembly stands in front of a currently unsolved biological problems, which could decipher the structure-function relationship of telomere maintenance and telomerase action. The single-molecule approach has been used so far for studying dynamics of single telomeric proteins or only their interaction domains, which has provided valuable information on shelterin dynamics. However, simultaneous interactions and the subsequent formation of several full-length shelterin subunits still represent a largely unaddressed field, where applying ensemble measurements can tell us only the average behavior and omit the specific and most crucial principal insights of shelterin assembly. Here, we utilized the single-molecule approach based on FCCS to address how shelterin proteins cooperate. To our knowledge, this is the first single-molecule study deciphering the shelterin assembly of full-length telomeric proteins. Moreover, the outcomes highlight a potential weakest point in telomere maintenance, and the single-molecule technique served as a platform for other methodologically related studies. In particular, the FCS significantly contributed to addressing the practical impacts of D₂O in fluorescence-based methods.

Research article 1 is focused on the human shelterin assembly at the single-molecule level. We investigated the simultaneous interactions and cooperation of shelterin core units TIN2, TRF1, TRF2, and TPP1 by using the FCCS approach.

At first, we reconstituted the shelterin core proteins from *E. coli* and subsequently fluorescently labeled them. In the initial FCCS experiment, we addressed how the titration of unlabeled TRF1 affects the relative cross-correlation of the preformed dual-labeled TRF2-TIN2 complex. Interestingly, we observed a dramatic decrease of TRF2-TIN2 relative cross-correlation upon TRF1 titration, suggesting that TRF1 can replace or dissociate TRF2 from the TRF2-TIN2 complex.

Secondly, we reversed the arrangement in the FCCS experiment and tested if the preformed dual-labeled TRF1-TIN2 complex stays intact upon TRF2 titra-

tion. Indeed, the relative cross-correlation level of TRF1-TIN2 remained unchanged, suggesting that TRF2 has no evident effect on the TRF1-TIN2 complex.

To complement the results, we carried out MST measurements confirming TRF1's higher affinity towards TIN2 than TRF2. Additionally, we employed FA to assess the binding properties of TRF1 and TRF2, when complexed with TIN2, to telomeric DNA. The obtained data revealed similar DNA binding affinities of TRF1-TIN2-TRF2 and TRF1-TIN2 complexes. Thus, TRF2 did not alter the DNA binding properties of the TRF1-TIN2 complex.

Next, we incorporated into the study another shelterin core subunit - TPP1. We repeated the arrangement of the first FCCS experiment, where we used dual-labeled TRF2-TIN2. Here, we preincubated the TIN2 protein with TPP1 prior to the mixture with TRF2. Then, we monitored the TRF2-TIN2 relative cross-correlation changes upon TRF1 titration in TPP1 presence. Strikingly, the TRF2-TIN2 relative cross-correlation remained at high levels upon TRF1 addition, which suggested that TRF2 was able to bind TIN2-TPP1 and tolerate TRF1 presence.

We summarized the proposed model in the final FCCS experiment (Figure 6.1). We carried out a study with dual-labeled TRF1 and TRF2, where their initial relative cross-correlation was in the level of the negative control. After adding TIN2 alone, the amplitude of relative cross-correlation between TRF1 and TRF2 did not change significantly. In contrast, the addition of preformed TIN2-TPP1 complex into the mixture of TRF1 and TRF2 significantly increased the relative cross-correlation between TRF1 and TRF2. Therefore, we confirmed the formation of the TRF1-TIN2-TPP1-TRF2 complex.

To strengthen the shelterin core assembly model, we conducted the FCCS measurements using two variants of TIN2 mutants (A15R and A110R). The A15R TIN2 mutant is unable to bind TPP1, and the A110R TIN2 mutant, on the other hand, can not bind TRF2. In our conditions, both TIN2 mutant variants prevented the formation of the whole shelterin core complex, which supports the essential role of TPP1 in expanding the TIN2 binding capacity and allowing TIN2 to accommodate both TRF1 and TRF2 simultaneously.

We also performed an ensemble measurement utilizing SEC to record the profiles of TRF1-TIN2-TRF2 mixture with or without TPP1 addition. We found that only in the TPP1 presence we can detect a significant high-molecular fraction corresponding to assembled shelterin core complex. Additionally, we characterized the collected fractions by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and DLS to verify the complex formation.

In review article 1, we summarized the recently published structural and quantitative data dedicated to shelterin and telomerase and discussed them in terms of the weakest point in telomere maintenance that could be utilized for possible

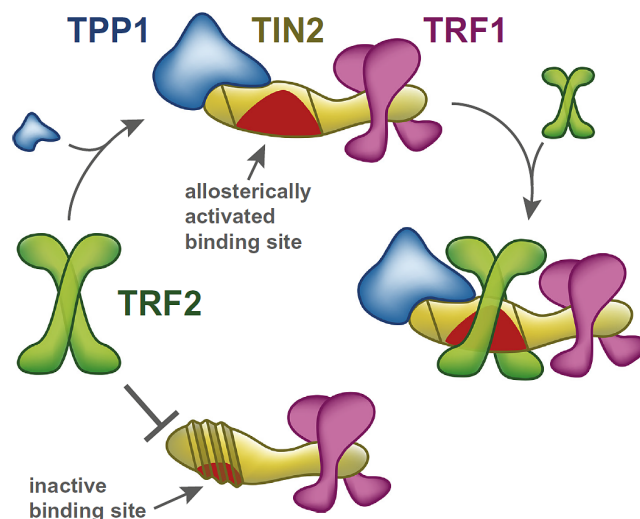


Figure 6.1: Proposed model describing the assembly of shelterin core subunits. In solution, TRF1 prevents the TRF2 from binding to TIN2 when TPP1 is absent. On the other hand, upon TPP1 complexation with TIN2, TPP1 enhances the binding capacity of TIN2. Thus, TIN2 can accommodate both TRF1 and TRF2 simultaneously [176].

anticancer strategies.

At first, we described the shelterin structure and mutual binding features of its subunits. We focused on shelterin assembly as an important factor for proper telomere maintenance. Furthermore, we illustrated the quantitative interaction map within shelterin and discussed the limiting step in shelterin complexation. According to the binary interaction studies, the weakest binding affinity within shelterin belongs to the TIN2-TPP1 interaction. With the support of achieved results from research article 1, where the TPP1 complexation with TIN2 is supposed to be crucial for stable shelterin core formation, we propose the role of TPP1, more precisely its interaction with TIN2, as the potential weakest point in telomere maintenance from a shelterin assembly perspective.

From a telomerase point of view, we described recent insights focused on the structure and function of telomerase, together with its recruitment via the shelterin protein TPP1. In addition, we discussed the inhibition of telomerase activity in the context of anticancer drug design. Two approaches involving inhibition of the TR-binding region of telomerase by GRN163L (Imetelstat), or hybridization of single-stranded telomeric overhang with T-oligonucleotides, respectively, have

been described.

Finally, we discussed the perspectives in finding the weakest point in telomere maintenance that could be utilized for possible cancer treatment. Here, we highlighted the importance of TPP1 protein, which stands in the center of shelterin assembly and telomerase recruitment (Figure 2.1a). Taken together, the TPP1 is involved in two essential events responsible for the proper telomere maintenance. Thus, TPP1 and its interactions with TIN2 and telomerase are promising candidates for a novel anticancer strategy.

In research article 2, we utilized the established FCS methodology, together with other microscopic and spectroscopic approaches, to characterize the practical implications of deuterated water on enhancing the fluorescence properties (Figure 6.2).



Figure 6.2: The enhancement of luminescence signal by deuterated water. The set of fluorophores is diluted in H_2O (left) or D_2O (right) buffers, respectively, and excited by ultraviolet light. Fluorophores from left to right: fluorescein, doxorubicin, rhein, ethidium bromide, propidium iodide [177].

We analyzed several fluorescent dyes using a single-molecule technique FCS and measured their counts per second per molecule (CPM) values in respective H_2O and D_2O buffers. We found that the average increase in CPM of all analyzed fluorophores is approx. 50%. The increase in CPM could significantly improve FCS measurements, since the CPM value can be viewed as a signal-to-noise ratio (SNR). In addition, the concentration used for standard FCS is extremely low in order to achieve single-molecule precision. Hence, a higher CPM enables using effectively the lower abundance of fluorophores.

Next, we applied wide-field fluorescence microscopy to visualize the difference in the brightness of either fixed or living cells stained samples after treatment with H_2O and D_2O buffers. We performed an indirect IF to detect β -actin to-

gether with DAPI staining of nuclei in fixed HEK293T cell samples. For live-cell imaging, we used HEK293T cells transiently expressing the fluorescent protein mCherry. In both cases, we observed an increase of approx. 30% in brightness, corresponding to the recorded steady-state measurements. The deuterated buffer enables lower excitation power, which is essential in avoiding photobleaching and preserving the viability of living cells.

HEK293T cells transiently expressing mCherry were also used to analyze the fluorescence lifetime changes after treatment with H₂O and D₂O buffers by FLIM. We showed that using the deuterated buffer prolongs the fluorescence lifetime by 37% on average. The longer fluorescence lifetime has an advantage in providing better imaging contrast and can lower the demand for the laser pulse width.

For super-resolution imaging, we discussed the possible benefits of the employment of D₂O. Here, the techniques are extremely dependent on the stability and brightness of used fluorophores. Additionally, the sample preparation is not trivial, and the measurements are generally time demanding. Therefore, incorporation of D₂O in super-resolution microscopy could seriously enhance the quality of performed experiments since it can increase the SNR, lower the excitation power, and preserve the viability of the cells.

Review article 2 is focused on viral transcriptional regulators (vTRs) and their structural and functional implications for anticancer therapy as a possible new direction of research in our laboratory.

We reviewed vTRs of three human oncoviruses. Firstly, we discussed the HBx (HBV X) protein of HBV (hepatitis B virus) as an essential factor promoting viral transcription and modulating several key host signaling pathways during viral infection. Next, we described the HBZ (HTLV-1 bZIP) factor of HTLV-1 (human T-cell lymphotropic virus 1), which indirectly affects host cell signaling and transcriptional pathways, ultimately mediating oncogenesis. Finally, we focused on the Rta factor of EBV, where we discussed its significance in activating EBV early genes and triggering the viral lytic state.

Overall, we rationalized the prospects of vTRs for potential antiviral-anticancer therapy. As vTRs play a crucial role in the viral life cycle, they can also alter various host cell signaling pathways and modulate transcription, ultimately leading to oncogenesis. The highlighted functions place vTRs as attractive targets for developing novel antiviral strategies.

7 DISCUSSION

Telomere maintenance presents a highly complex system that covers various processes involved in regulating telomere length and protecting chromosome ends. Shelterin orchestrates essential actions that shape the telomeric structure, guard telomeres against DDR, and directly mediate the telomerase association to telomeres. The assembly of shelterin can regulate the functions of the whole complex. Therefore, shelterin assembly tackles several outstanding biological questions regarding telomere maintenance. This work is focused on the shelterin core formation and addresses the molecular assembly mechanism at the single-molecule level. Together with the FCCS-based single-molecule platform, the findings were discussed and reviewed individually in appended articles. Here, the aim is to put into perspective new articles published during the writing of the thesis and to discuss the prospects of future directions.

Our study strengthens the understanding of how shelterin core proteins cooperate. In particular, we contributed to describing the role of the TPP1 subunit in promoting higher-order shelterin complexation and suggest the order preference during assembly. The already published structural data revealing the TRF2-TIN2 interface upon TPP1 binding supports the model (Figure 6.1) that the TRF2 binding site on TIN2 is activated when TPP1 binds to TIN2 [70]. Additionally, the binding affinity of TRF2 to TIN2, measured by fluorescence polarization, increases up to 3-fold when TIN2 is complexed with TPP1 [70]. Interestingly, another study observed an even 18-fold increase in binding affinity in the same arrangement measured by isothermal titration calorimetry [178]. Thus, TPP1 binding indeed induces structural changes in TIN2, expanding its binding capacity and enabling TIN2 to accommodate both TRF1 and TRF2 simultaneously.

At this moment, the structure of DNA-bound telomerase in complex with TPP1-POT1 heterodimer has been resolved by cryo-EM [179]. The fascinating result shed new light on the molecular mechanism of telomerase recruitment to telomeres. Sekne *et al.* confirmed the direct involvement of NOB and TEL-patch regions of TPP1 and demonstrated their three contact areas with TERT [46,

179]. Moreover, the detailed interaction surface from the TERT perspective was mapped, which solved the long-standing structure-function-related question in the telomere maintenance field [123, 179]. Sekne *et al.* also identified a specific motif in the TEN domain of TERT, which binds the telomeric DNA substrate and turns it towards the TEN-POT1 interface. The identified motif confirmed the previously proposed DNA anchor site [32, 179]. Taken together, the study underlines the importance of shelterin subunit TPP1 and supports the hypothesis as a potential weakest point in telomere maintenance. It would be of great importance to utilize the published structural data to design specific small molecule inhibitors targeting the TPP1-telomerase interface or TPP1-TIN2 interaction as a novel anticancer strategy.

A newly reported study focused on the reconstitution of the entire shelterin complex highlights the extreme structural heterogeneity and dynamics of shelterin units [144]. The observed flexibility of shelterin components prevented the detailed structural characterization. However, Zinder *et al.* confirmed the shelterin's ability to assemble without telomeric DNA substrate, supporting our results and the proposed model (Figure 6.1) of shelterin core formation. Another recent work complements the DNA binding properties of the two OB-folds of POT1, which are flexibly tethered to TPP1 and therefore can function with high conformational versatility [144, 180]. Interestingly, the new structural data of DNA-bound telomerase in complex with TPP1-POT1 heterodimer resolved only the N-terminal part of TPP1 containing OB-fold and the two N-terminal OB-folds of POT1, even though the full-length proteins with also TIN2 addition were used [179]. Thus, more independent studies directly contribute to the high heterogeneity of the shelterin complex. The dynamic aspect of shelterin composition, together with underappreciated features mainly involving long flexible regions of shelterin units, has been recently reviewed [23]. Lim *et al.* cited our work dedicated to shelterin core assembly at the single-molecule level, and they stressed the importance of studying the shelterin functions and dynamics in the context of complexes instead of individual proteins or isolated domains [23].

The recently emerged mechanism based on liquid-liquid phase separation (LLPS) brought valuable insights into controlling telomere maintenance. LLPS tends to describe the formation and behavior of membrane-less organelles, which exhibit a higher local density of proteins and nucleic acids that create condensates with liquid-like characteristics. Novel studies described telomeres in cells as a multicomponent phase-separated liquid where telomeric DNA and shelterin complex serves as a scaffold. Moreover, TRF1 and TRF2 subunits drive the coalescence of shelterin and modulate the phase boundaries, which regulate the access of other factors into telomere condensates. The shelterin stoichiometry has to be dynam-

cally balanced to the telomere length in order to control the formation of telomere condensation [181, 182]. Overall, the LLPS model creates an additional view that helps understand the mechanisms involved in telomere maintenance.

In summary, the results presented in this work contribute to understanding how shelterin subunits cooperate. We extended the current knowledge of shelterin dynamics by employing a single-molecule approach based on FCCS with full-length telomeric proteins. Despite the novel research additions to shelterin architecture, the overall complex formation and mechanisms of shelterin dynamics remain largely unknown. Most importantly, the fundamental challenge is to probe the shelterin architecture and describe the dynamics of shelterin within the complex environment of living cells.

8 FINAL REMARKS

Telomere maintenance is essential for ensuring genome integrity and stability. Dysfunction in telomere length regulation is associated with diseases such as bone marrow failure, premature aging, and cancer. Human telomeric DNA is bound by the shelterin complex, which acts as a telomere guardian. Shelterin protects the telomeres against unwanted DDR machinery and directly recruits telomerase to the telomeres. In addition, shelterin regulates the access of other factors to telomeric DNA. The molecular mechanism of how shelterin is assembled tackles an outstanding biological question, which would reveal crucial information about telomere maintenance. This thesis is focused on the elucidation of human shelterin assembly at the single-molecule level.

We utilized the FCCS to describe the formation of core shelterin proteins TRF1, TRF2, and TIN2. We found that TRF1 can effectively exchange TRF2 bound to TIN2. On the other hand, the addition of TRF2 to the preformed TRF1-TIN2 complex had no evident effect. Next, we examined the role of shelterin factor TPP1 in promoting whole shelterin complexation. We showed that TPP1, upon binding to TIN2, enhances TIN2 binding ability and enables TIN2 to accommodate both TRF1 and TRF2 simultaneously. Thus, TPP1 is essential in stable shelterin core formation. Based on our findings and already published structural data, we proposed a mechanistic model describing the shelterin core assembly. I am honored that I could present the main results of shelterin core assembly in the form of a selected talk at the prestigious EMBO conference dedicated to telomere biology in health and human disease.

Furthermore, we reviewed the shelterin factor TPP1 as the potential weakest point in telomere maintenance. Since TPP1 is directly responsible for telomerase recruitment to telomeres and the TPP1-TIN2 interaction promotes the whole shelterin assembly, TPP1 is essential in two critical events regarding telomere length regulation.

The thesis contributes to understanding how the shelterin core proteins are assembled in solution at a single-molecule level. In addition, the FCS technique

was utilized in methodologically related studies, where we characterized the practical effects of deuterated water on the enhancement of fluorescence signals.

Research in telomere biology has recently shown fascinating findings. The structure of telomerase bound to telomeric DNA and TPP1-POT1 heterodimer was revealed [179], the endogenously tagged telomerase and individual shelterin units can be monitored in living cells [47], and the dynamics and extensive structural heterogeneity of shelterin complex have been proposed [144]. As the new advances in cutting-edge techniques like super-resolution fluorescence microscopy, LLPS, and cryo-EM emerge, future studies will be necessary to further elucidate such a complex mechanism of telomere maintenance. Therefore, new insights into the regulation of telomere length would be essential in developing therapies against telomere-associated diseases.

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APPENDIX

Curriculum vitae



Tomas Janovic

PhD Candidate at
CEITEC - Central European
Institute of Technology and
Masaryk University

Date of Birth: Nov. 14, 1989

Oldrichovice 56, 739 61, Trinec,
Czech Republic

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janovic.tom@gmail.com

Lifeb laboratory

ORCID: 0000-0003-4471-5756

Interests

- Advanced Microscopy Techniques
- Telomere Biology
- Image and Signal Processing
- Dynamics of Protein Complexes
- Single-molecule Biophysics

Skills

Laboratory skills:

Microscopy

Fluorescence methods

Wet Lab

Genomics

Proteomics

Cell Culture

Programming:

MATLAB, C#

VBA, C++, R

Software:

MS Office

Adobe Photoshop

LaTeX - Overleaf

Working Experience

- Jan, 2019 –
ongoing

Research Fellow Masaryk University, National Centre for Biomolecular Research, Lifeb laboratory, Brno, Czech Republic

Studying the molecular mechanisms of telomerase inhibition, shelterin assembly, and REST-TRF2 complex formation and function. Focused on protein-protein and protein-DNA interactions primarily by using advanced microscopy techniques.
- Jun, 2015 –
Sep, 2018

Process Engineer Honeywell, Inc., Brno, Czech Republic

Validation and implementation of new electronic products into the production. Managing ECR/O processes. Automation of label printing with programming an application for ordering and printing labels. Creating database for storing all label's data and link them into the layouts. Creating VBA macros for production support. Implementation of Lean & Six Sigma methods for production optimization.
- Sep, 2014 –
May, 2015

Trainee Honeywell, Inc., Brno, Czech Republic

Practical introduction into the company's operations. Preparing for the next position as a process engineer. Two months of experience in Germany during the material/production transfer.
- Jul, 2013 –
Aug, 2013

Internship Institute of Scientific Instruments CAS, Brno, Czech republic

Medical signal processing, study the problematic of QT-RR interval.

Education

Postgraduate Studies

- 2016 –
ongoing

Ph.D. in Bio-omics CEITEC - Central European Institute of Technology and Masaryk University, Brno, Czech Republic

Title: Human Shelterin Assembly and Transcription Regulation.

Supervisor: Assoc. Prof. Ctirad Hofr, Ph.D.

Single-molecule

FCCS

Shelterin assembly

Lifeb
- 2012 – 2014

M.Eng. in Biomedical Engineering Brno University of Technology, Czech Republic

Title: Contour Shape Classification For Detection of Mis-segmented Bones in CT Data.

Supervisor: Ing. Petr Walek, Ph.D.

Grade: Passed with honour

Wavelet transform

Shape descriptors

Segmentation

CT

Undergraduate Study

- 2009 – 2012

B.Sc. in Biomedical Technology and Bioinformatics Brno University of Technology, Czech Republic

Project Title: Detection of Eyes in Facial Images by Means of Hough Transform.

Supervisor: Ing. Petr Walek, Ph.D.

Other Training

- Oct 2019

Instruct-ERIC Workshop on Computational Approaches in Integration of Structural Biology Techniques BIOCEV, Vestec, Czech Republic

Gained practical experience with advanced MS, biophysical methods, crystallization, and X-ray diffraction and SAXS.
- Oct 2018

Single Molecule Microscopy and Manipulation BIOCEV, Vestec, Czech Republic

Training on single-molecule methods utilizing FCS and TIRF.
- Jun 2017

Advanced Fluorescence Imaging Techniques EMBL, Heidelberg, Germany

Training on fluorescence microscopy advanced techniques including FRET, FRAP, SMLM, TIRF, Expansion microscopy, Deconvolution.
- Jun 2017

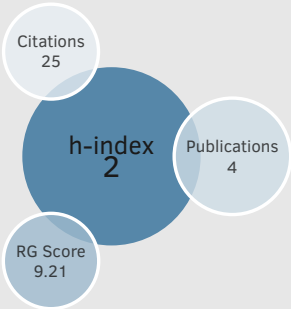
Summer School in New Approaches in Experimental Biology and Omics CEITEC, Brno, Czech Republic

Obtaining theoretical knowledge and practical skills in modern techniques used in gene and protein analyses.

Short Bio

Tom received his master's degree from biomedical engineering at the Brno University of Technology, Czech Republic in 2014, where he focused on signal and image processing techniques. Then he pursued his career as a process engineer in Honeywell, Inc. Currently, he is a Ph.D. candidate at CEITEC, Masaryk University in Brno, Czech Republic. He is part of a Lifeb team, where he investigates human nucleoprotein complexes by using advanced fluorescence microscopy techniques.

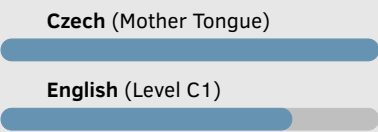
Metrics



Profiles



Languages



Personal

Tom is an open-minded person who tries to view the world positively and with a smile on his face. From his childhood, when he played ice hockey, he has maintained an important team spirit. Tom still loves sport, especially rock-climbing, trail-running, and hiking. He is also a passionate photographer specializing in outdoor and landscape photography (Gallery), but he also photographed several weddings. Tom always finds time to spend with his family and friends. He loves his girlfriend and enjoys a good cup of green tea or coffee.

Teaching Experience

Postgraduate Courses

CEITEC	Advanced Fluorescence Imaging Course on FLIM and FCS Spring'19 Fluorescence Cross-correlation Spectroscopy and data interpretation.
CEITEC	Talking Colors: Advanced Microscopy Fall'17 Fluorescence Cross-correlation Spectroscopy. Giving practical lecture together with Prof. Michael D. Stone and Dr. Mark Hink.

Undergraduate Courses

Masaryk University	C7235: Fluorescence methods in life sciences Fall'16 - Fall '21 Fluorescence Microscopy, FRET, Protein Labeling, Protein Analyses, Fluorescence Spectroscopy, Fluorescence Anisotropy.
Masaryk University	IBIS - Interactive Biological Seminar 2019-2021 Participated in teaching talented high school students the basics of biophysical methods used in life sciences.

Academic Supervision

2020–2022	DNA Binding Mechanism of Telomeric Proteins Protecting Human Chromosomes.	M.Sc.
2020–2022	Quantitative Description of Interactions of Telomere Proteins and Transcription Factors.	M.Sc.
2019–2020	Interactions of Telomeric Proteins That Are Essential For Human Genome Stability.	B.Sc.
2018–2019	Interactions of Human Telomeric Proteins That Protect Chromosomes.	B.Sc.

Honours and Awards

Feb 2021	2 nd Place at CEITEC PhD Conference in Poster Category.	CEITEC, CZE
Sep 2016	Awarded CEITEC Scholarship.	CEITEC, CZE
Jun 2014	Dean's Award of Master's Thesis.	Brno Univ. of Tech., CZE
Mar 2014	1 st Place at Student Competition EEICT in Category - Signal, Image and Data Processing.	Brno Univ. of Tech., CZE
Sep 2013	Awarded M.Sc. Scholarship.	Brno Univ. of Tech., CZE

References

Ref. 1	Assoc. Prof. Ctirad Hofr, Ph.D. Masaryk University, Brno, Czech Republic hofr@sci.muni.cz
Ref. 2	Prof. Jiri Fajkus, CSc. Masaryk University, Brno, Czech Republic fajkus@sci.muni.cz
Ref. 3	Prof. Jiri Jan, CSc. Brno University of Technology, Czech Republic jan@vut.cz

Publications

Journals

- Nečasová I., Stojaspal M., Motyčáková E., Brom T., Janovič T. & Hofr C., Transcriptional regulators of human oncoviruses – structural and functional implications for anticancer therapy. *NAR Cancer* **4**. ISSN: 2632-8674 (2022).
- Kučera J., Peš O., Janovič T., Hofr C., Kubinyiová L., Tóth J., Káňa Š. & Táborský P., Enhancement of Luminescence Signal by Deuterated Water – Practical Implications. *Sensors and Actuators B: Chemical* **352**. ISSN: 0925-4005 (2022).
- Veverka, P., Janovič, T. & Hofr, C. Quantitative Biology of Human Shelterin and Telomerase: Searching for the Weakest Point. *International Journal of Molecular Sciences* **20**. ISSN: 1422-0067 (2019).
- Janovič, T., Stojaspal M., Veverka, P., Horáková D. & Hofr, C. Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 when TPP1 Is Absent. *Journal of Molecular Biology* **431**. ISSN: 0022-2836 (2019).

Conferences

- PicoQuant 26th International Workshop on “Single Molecule Spectroscopy and Super-resolution Microscopy”, Berlin, Germany, 2021 - **Poster**: Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 when TPP1 Is Absent.
- CEITEC PhD Conference, Brno, Czech Republic, 2021 - **Poster**: Assembly of Human Guardians of Telomeres: TRF1 Exchanges TRF2 Bound to TIN2 but Tolerates TRF2 in TPP1 Presence.
- CEITEC PhD Conference, Brno, Czech Republic, 2020 - **Selected Talk**: Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 when TPP1 Is Absent.
- EMBO Workshop, Telomere biology in health and human disease, Tróia, Portugal, 2018 - **Selected Talk**: Human TRF1 Exchanges TRF2 Bound to TIN2 but Tolerates TRF2 in TPP1 Presence.
- CECE Conference, 15th International Interdisciplinary Meeting on Bioanalysis, Brno, Czech Republic, 2018 - **Poster**: Human TRF1 Exchanges TRF2 Bound to TIN2 but Tolerates TRF2 in TPP1 Presence.
- Student EEICT Conference, Brno, Czech Republic, 2014 - **Selected Talk**: Contour Shape Classification for Detection of Mis-Segmented Bones in CT Data.

Research article 1

Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 When TPP1 Is Absent

T. Janovič, M. Stojaspal, P. Veverka, D. Horáková, C. Hofr
Journal of Molecular Biology (2019), DOI: [10.1016/j.jmb.2019.05.038](https://doi.org/10.1016/j.jmb.2019.05.038)



Human Telomere Repeat Binding Factor TRF1 Replaces TRF2 Bound to Shelterin Core Hub TIN2 when TPP1 Is Absent

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<https://doi.org/10.1016/j.jmb.2019.05.038>

Edited by Titia Sixma

Abstract

Human telomeric repeat binding factors TRF1 and TRF2 along with TIN2 form the core of the shelterin complex that protects chromosome ends against unwanted end-joining and DNA repair. We applied a single-molecule approach to assess TRF1–TIN2–TRF2 complex formation in solution at physiological conditions. Fluorescence cross-correlation spectroscopy was used to describe the complex assembly by analyzing how coincident fluctuations of differently labeled TRF1 and TRF2 correlate when they move together through the confocal volume of the microscope. We observed, at the single-molecule level, that TRF1 effectively substitutes TRF2 on TIN2. We assessed also the effect of another telomeric factor TPP1 that recruits telomerase to telomeres. We found that TPP1 upon binding to TIN2 induces changes that expand TIN2 binding capacity, such that TIN2 can accommodate both TRF1 and TRF2 simultaneously. We suggest a molecular model that explains why TPP1 is essential for the stable formation of TRF1–TIN2–TRF2 core complex.

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Introduction

Human telomeres are maintained by telomerase [1,2] and protected by telomeric proteins [3,4]. Telomeric proteins recruit telomerase to telomeric DNA [5]. Shelterin is a six-protein complex comprising TRF1, TRF2, TIN2, TPP1, POT1 and RAP1. Shelterin associates specifically with telomeric DNA repeats and protects linear chromosome ends from being recognized by the DNA repair machinery as damaged DNA [4]. TRF1 and TRF2 (telomere repeat-binding factor 1 and 2) bind the double-stranded telomeric DNA [6,7]. TRF2 protects chromosomes by forming lasso-like structures through the invasion of the 3' single-stranded overhang into the duplex telomeric repeats [8,9] while suppressing ATM activity [10]. RAP1 interacts solely with TRF2 and regulates the specific binding of TRF2 to telomeric DNA and subsequent telomeric loop processing by helicases [11,12]. TIN2 (TRF1-interacting nuclear factor 2) [13] binds both factors, TRF1 and TRF2 [4]. In addition, TIN2 recruits TPP1

("TPP1" combines the first letter of each name, TINT1 [14], PTOP [15] and PIP1 [16], from the three groups that initially characterized the human protein). TPP1 forms a heterodimer with POT1 (protection of telomeres 1) [17]. From the structural point of view, TIN2 is the central hub of the shelterin complex that links TRF1 and TRF2 homodimers with TPP1–POT1 heterodimers. The domains of TIN2 that take part in the interaction with TRF1, TRF2 and TPP1 are shown in Fig. 1a.

Regarding its biological functions, TIN2 is essential for telomere length regulation mediated by TRF1 [21,22]. TIN2 is required for TRF2-induced protection against ATM signaling pathway [23] and POT1-mediated protection against ATR signaling pathway [24,25]. In cells, TIN2 deletion compromises the stability of both TRF1 and TRF2 at telomeres [13,26]. TIN2 bridges TRF1 and TRF2 with TPP1, which then recruits telomerase to telomeres [5,27] and enhances telomerase processivity upon complexation with POT1 [28–30]. TIN2 is expressed in two isoforms with different biochemical and

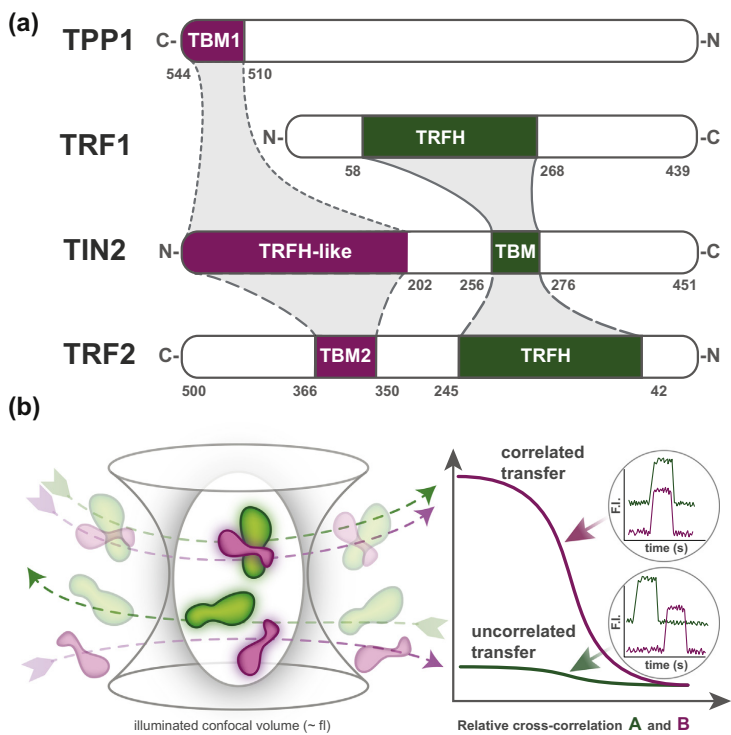


Fig. 1. TIN2 mediates the assembly of shelterin core proteins—interaction domains interconnect TIN2 with TRF1, TRF2 and TPP1. (a) Scheme of the studied proteins showing the interaction domains: TBM1, TIN2-binding motif of TPP1; TRFH, dimerization domain of TRF1 and TRF2; TRFH-like, dimerization domain of TIN2; TBM, TRFH of TRF1/TRF2 binding motif of TIN2; TBM2, TIN2-binding motif of TRF2. The more solid the outline stroke between the interacting domains, the higher their mutual affinity [18,19]. All identified interacting domains of shelterin proteins have been recently reviewed in Ref. [20]. (b) Scheme of how FCCS detects mutually bound proteins. When a green fluorescently labeled protein A and a red fluorescently labeled protein B diffuse through the illuminated confocal volume, fluorescence signals fluctuations are recorded simultaneously. If A binds B, the proteins move together, so they produce fluorescence intensity fluctuations of similar patterns for both fluorescence labels and the cross-correlation amplitude increases accordingly.

functional patterns [31]. Mutations in the gene of TIN2 have been implicated in approximately 15% of all known cases of *dyskeratosis congenita*—a disease that results in defective telomere maintenance in early childhood [32,33]. The assembly of shelterin subunits around TIN2 is critical for the formation of structurally and biologically functional shelterin complexes. The overall shelterin protein ratios on telomeres are known from *in vivo* experiments [34]. Newly, the *in vitro* stoichiometry of an assembled core complex comprising TRF2, TIN2, TPP1 and POT1 was revealed to be 2:1:1:1, respectively [35].

Previous studies described the structure and binding affinity of peptides representing interaction regions that take part in TRF1 and TRF2 binding to

TIN2 [18]. Very recently, the structure of the isolated interacting domains of TRF2, TIN2 and TPP1 has been determined [19].

Furthermore, Hu *et al.* [19] postulated how structural changes in the TRFH-like domain of TIN2 (2–202) upon association with TPP1 domain TBM1 (544–510) increase the binding affinity of the TBM2 domain of TRF2 to TIN2. In addition, Kim *et al.* used isothermal titration calorimetry to reveal that the complexation of full-length TIN2 with TPP1 fragment (486–544), containing TBM1 domain, promotes association of TRF2 fragment (382–424) and TIN2 [36].

Despite the newly revealed structural–function relationships within shelterin at the domain level, very little is known about how full-length TRF1, TRF2

and TIN2 affect each other and TPP1 during shelterin assembly. The quantitative studies of shelterin proteins moving freely in solution represent an experimental challenge connected with the comparable size of TRF1 and TRF2 that excludes using simple fluorescence polarization measurements. Single-molecule approaches are powerful tools of assessing the functional states of a molecular system as has been demonstrated by assessing DNA-repair complex assembly and dynamics [36,37] along with mechanistic insights into telomeric proteins and telomerase function [38]. Nevertheless, classical single-molecule total internal reflection fluorescence microscopy is often limited to the area near the surface, where studied molecules are attached. Instead, by means of confocal scanning microscopy, we can measure interactions of fluorescently labeled proteins moving freely in solution regardless of the distance from the surface.

In this study, we took advantage of fluorescence cross-correlation spectroscopy (FCCS)—a single-molecule method that is based on an evaluation of the interdependence of time-resolved fluctuations of two different fluorophores by confocal microscopy [39]. FCCS monitors simultaneous fluorescence signals of two differently labeled proteins that diffuse through the confocal volume of a microscope objective (Fig. 1b) [40,41]. In particular, FCCS has been extensively used to describe the assembly of oligomeric calcium/CaM-dependent kinase II and calmodulin by the Schwille laboratory [39].

We used FCCS to monitor protein interactions *in vitro* based on the change in relative cross-correlation of differently labeled TIN2, TRF1 and TRF2 and to address the following hypotheses. First, we wanted to know whether both TRF1 and TRF2 bind TIN2 simultaneously or if there is an order preference during the shelterin subcomplex assembly. In addition, we tested the hypothesis that TPP1 binding to TIN2 may improve TRF2–TIN2 interaction and could enable TIN2 to interact simultaneously with TRF1 and TRF2, as has been suggested by the Songyang laboratory [42]. Finally, we wondered if we could suggest an interaction model of TRF1, TRF2, TIN2 and TPP1 assembly and correlate the model with available structural data and biological functions of shelterin proteins.

We found that TRF1 induces TRF2 release from TIN2. We also described that TPP1, upon binding to TIN2, improves TIN2's binding capacity so the complex TIN2–TPP1 can accommodate both TRF1 and TRF2. We showed for the first time with full-length TRF1 at the single-molecule level that TPP1 is essential for the formation of the stable TRF1–TIN2–TPP1–TRF2 complex. We suggest a mechanism that explains the mutual exclusivity of TRF1–TIN2 and TRF2–TIN2 interactions along with the requirement of TPP1 for simultaneous binding of TRF1 and TRF2 to TIN2. This work is, to our knowledge, the first single-molecule study describing the assembly of full-length proteins TRF1, TRF2 and TIN2.

Results

TRF1 replaces TRF2 bound to TIN2

At first, we wanted to know whether full-length TIN2 is able to accommodate both full-length TRF1 and TRF2 simultaneously. We allowed forming complexes of TRF1–TIN2 and TRF2–TIN2 at micromolar concentrations as suggested by dissociation constants obtained from our microscale thermophoresis assay (Supplementary Fig. S5). The complexes TRF1–TIN2 and TRF2–TIN2 were prepared with 2:1 stoichiometry. Subsequently, the complex solutions were diluted to the concentration required for a single-molecule detection. For our FCCS measurements, TRF1 or TRF2 (20 nM) was labeled with red fluorophore Alexa Fluor 594 and TIN2 (10 nM) was labeled with the green fluorophore Alexa Fluor 488.

In the first experiment, we titrated dual-labeled TRF2–TIN2 complex with unlabeled TRF1 to a final concentration of 80 nM (Fig. 2a and b). Initially, when the TRF2–TIN2 complex was stably formed, we observed a high relative cross-correlation corresponding to the relative cross-correlation of the positive control (Supplementary Fig. S1). Thus, we can affirm that the TRF2–TIN2 complex was stably formed. Immediately after the addition of 2.5 nM of TRF1, we observed that the relative cross-correlation between TIN2 and TRF2 decreased to the level of the negative control (Supplementary Figs. S1 and S2). Overall, the decrease of relative cross-correlation suggested that TRF1 replaced TRF2 in the complex with TIN2 (Fig. 2a and b).

TRF2 showed no influence on TRF1–TIN2 complex

In the next sets of experiments, we used a reverse arrangement where TRF1 labeled with red fluorophore (Alexa Fluor 594) was allowed to bind TIN2 labeled with green fluorophore (Alexa Fluor 488). Subsequently, we added unlabeled TRF2 gradually and monitored if TRF2 can disturb the complex TRF1–TIN2. We measured the relative cross-correlation of labeled TRF1 and TIN2 at each concentration of unlabeled TRF2 (Fig. 2c and d). The relative cross-correlation of TRF1 and TIN2 remained high and stable at TRF2 concentration up to 80 nM. In other words, when we added TRF2 to the preformed complex TRF1–TIN2, we detected no significant decrease of relative cross-correlation between TRF1 and TIN2 (Supplementary Fig. S2). The minimal effect of TRF2 presence on TRF1–TIN2 relative cross-correlation suggested that TRF2 did not disturb the TRF1–TIN2 complex.

TRF2 has no effect on DNA binding affinity of TRF1–TIN2

We also wanted to assess whether the presence of TRF2 affects the binding of TRF1 to telomeric

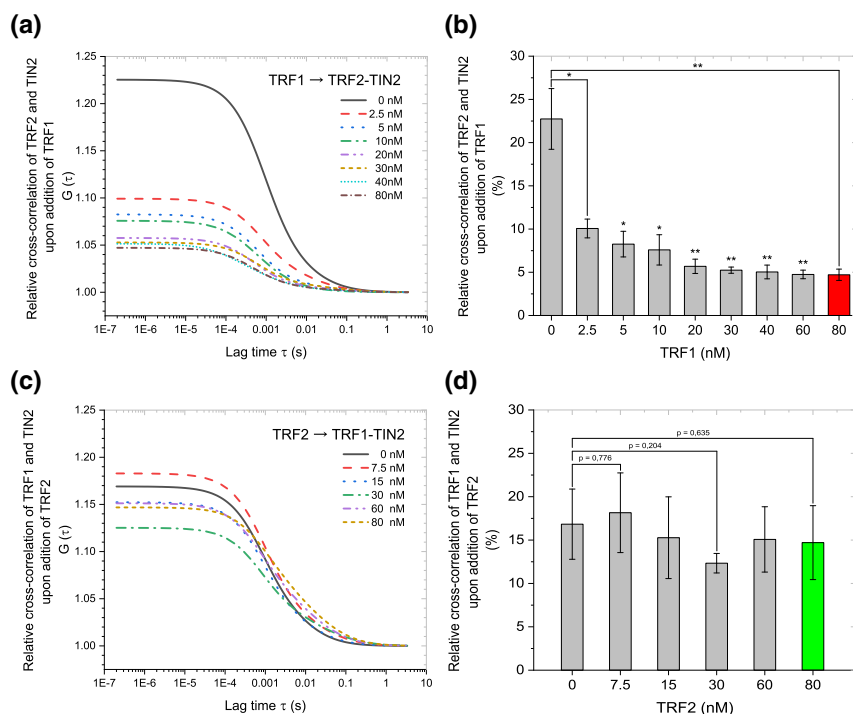


Fig. 2. TRF1 replaces TRF2 from TIN2, whereas TRF2 has no effect on TRF1–TIN2 complex. (a) The relative cross-correlation of fluorescently labeled TRF2 (20 nM) and TIN2 (10 nM) measured upon addition of unlabeled TRF1 (0–80 nM). Fits of relative cross-correlation curves upon increase of TRF1 show a decrease of the amplitude of TRF2–TIN2 relative cross-correlation. (b) The amplitudes of TRF2–TIN2 relative cross-correlation at 0–80 nM TRF1 presence—determined from panel a. Error bars represent standard deviations of three independent measurements. *P*-values: two-tailed Student's *t* test with regard to the amplitude without TRF1 (0 nM); **P* < 0.05, ***P* < 0.01. (c) The relative cross-correlation of fluorescently labeled TRF1 (20 nM) and TIN2 (10 nM) measured upon addition of unlabeled TRF2 (0–80 nM). Fits of relative cross-correlation curves upon increase of TRF2 show that the amplitude of TRF2–TIN2 relative cross-correlation stays at the initial level. (d) The amplitudes of TRF1–TIN2 relative cross-correlation at 0–80 nM TRF2 presence—determined from panel c. Error bars represent standard deviations of three independent measurements. *P*-values: two-tailed Student's *t* test with regard to the amplitude without TRF2 (0 nM).

DNA when TRF1 is in complex with TIN2. To analyze how mutual TRF1, TRF2 and TIN2 interactions affect DNA binding affinity, we employed fluorescence anisotropy (Fig. 3). For this purpose, we used a telomeric DNA duplex R5 containing five telomeric repeats and a stoichiometric combination of TRF1, TRF2 and TIN2, 2:2:1, respectively, as has been suggested previously [34,35]. R5 should feasibly accommodate both TRF1 and TRF2 simultaneously. Our quantitative binding data revealed that the DNA binding affinity of the stoichiometric combination of TRF1, TRF2 and TIN2 is similar to the DNA binding affinity of the combination TRF1 and TIN2. In other words, TRF2 did not affect the DNA binding affinity of TRF1–TIN2.

TPP1 enables TIN2 to accommodate both TRF2 and TRF1 simultaneously

We wondered whether another human telomeric protein TPP1 improves the stability of TRF1–TIN2–TRF2 complex consisting of full-length proteins. Hu *et al.* suggested that the C-terminal domain of TPP1 is responsible for its binding to TIN2 and that TPP1 stabilizes the TIN2–TRF2 interaction [19]. The full-length TPP1 purity was insufficient for single-molecule experiments; thus, recombinant human TPP1 with an N-terminal deletion was used. TPP1 (89–554) was chosen because the 88 N-terminal residues of TPP1 are functionally dispensable in human cells and are not conserved among TPP1

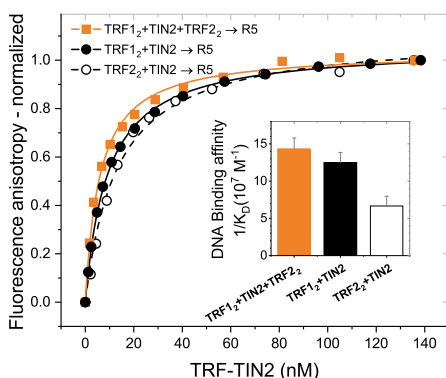


Fig. 3. DNA binding affinity of stoichiometric combination of TRF1/TIN2/TRF2 (2:1:2) is similar to DNA binding affinity of stoichiometric combination of TRF1/TIN2 (2:1). TRF1 (5 μ M) and/or TRF2 (5 μ M) was incubated with TIN2 (2.5 μ M) in 50 mM sodium phosphate (pH 7.0) and 50 mM NaCl at 25 $^{\circ}$ C. Protein solutions were titrated to Alexa Fluor 488-labeled telomeric DNA duplex R5 containing five telomeric repeats. The presented binding isotherms are averages of five independent experiments with standard deviation lower than 3% for each presented data point. The inset bar plot shows reciprocal K_D values that correspond to DNA binding affinity.

proteins of different organisms [16,30,43]. In addition, TPP1 (89–554) still contains the N-terminus of the OB domain that is critical for telomerase activity [44]. For simplicity, we hereafter use TPP1 to refer to TPP1 (89–554) unless stated otherwise.

We incubated fluorescently labeled TRF2 and TIN2 along with unlabeled TPP1 at room temperature. Then, we added unlabeled TRF1 to see whether TRF1 can still remove TRF2 from the TIN2–TPP1–TRF2 complex. As Fig. 4a shows, relative cross-correlation between TRF2 and TIN2 remained at high levels for all concentrations of TRF1. The relative cross-correlation between TRF2 and TIN2 was changed insignificantly according to calculated P -values (Fig. 4b). The statistically insignificant change of relative cross-correlation suggested that the majority of TRF2 remains bound to TIN2–TPP1 in the presence of TRF1.

The formation of the complex TRF1–TIN2–TPP1–TRF2 was confirmed by a complementary FCCS experiment where TRF1 and TRF2 were labeled by Alexa Fluor 488 and Alexa Fluor 594, respectively. First, unlabeled TIN2 was added to the mixture of labeled TRF1 and TRF2. After TIN2 addition, we observed no significant change in the amplitude of relative cross-correlation between TRF1 and TRF2 (Fig. 4c). On the contrary, when we added preformed

complex of TIN2–TPP1 into TRF1 and TRF2 mixture, we detected a substantial increase of relative cross-correlation between TRF1 and TRF2 (Fig. 4c).

When we mixed TRF1, TIN2, TRF2 and TPP1 together, we observed high relative cross-correlation between TRF1 and TRF2, similar to the relative cross-correlation between TRF2 and TIN2 in the previous experimental setup (compare Fig. 4a and c). The high relative cross-correlation level in both experimental arrangements verified that the TRF1–TIN2–TPP1–TRF2 complex was formed.

To confirm that the proteins form a stable complex, we recorded size-exclusion chromatography profiles of a mixture comprising TRF1, TIN2 and TRF2 with or without TPP1 (Fig. 4d). Only in the presence of TPP1 we observed a high-molecular peak that corresponds to the assembled protein complex (compare the solid red line and dashed blue line in Fig. 4d). We analyzed collected chromatographic fractions by SDS gel electrophoresis. As TRF1 and TRF2 were labeled by different fluorophores, we identified electrophoretic bands corresponding to TRF1 and TRF2 (insets in Fig. 4d). The fluorescence intensity profiles showed that TRF1 and TRF2 formed a complex only if TPP1 and TIN2 were present. Furthermore, the collected fractions were characterized by dynamic light scattering to verify that TRF1–TIN2–TRF2–TPP1 complex was formed (Fig. S9).

In addition, we have carried out control measurements with TIN2 mutants that were unable to bind TPP1 or TRF2. We have prepared two TIN2 point mutants—A15R and A110R. The A15R mutation inhibits TPP1 binding and the A110R mutation inhibits TRF2 binding to the N-terminus of TIN2, as revealed by Hu *et al.* [19]. Our FCCS measurements showed that both mutations of TIN2 restricted the assembly of the shelterin core complex (Supplementary Fig. S4). We found that TIN2 A110R was unable to form complex with TRF2 even in the presence of TPP1 and TRF1 (Supplementary Fig. S4a).

The inability of TIN2 A110R to bind TRF2 indicates that N-terminal binding site of TIN2 is critical for the stable accommodation of TRF2 into the complex. FCCS measurements with the second mutant revealed that TIN2 A15R, with impaired TPP1 binding ability, was unable to cross-correlate with TRF2 in the presence of TRF1 and TPP1 (Supplementary Fig. S4b). Thus, FCCS experiments with TIN2 mutants supported the view that the N-terminal domain of TIN2 is essential for the cooperative binding of TRF2 and TPP1 to TIN2. In summary, our combined single-molecule and ensemble analyses of shelterin core proteins suggest that TPP1 enables TIN2 to bind both TRF1 and TRF2 simultaneously and form stable TRF1–TIN2–TPP1–TRF2 complex.

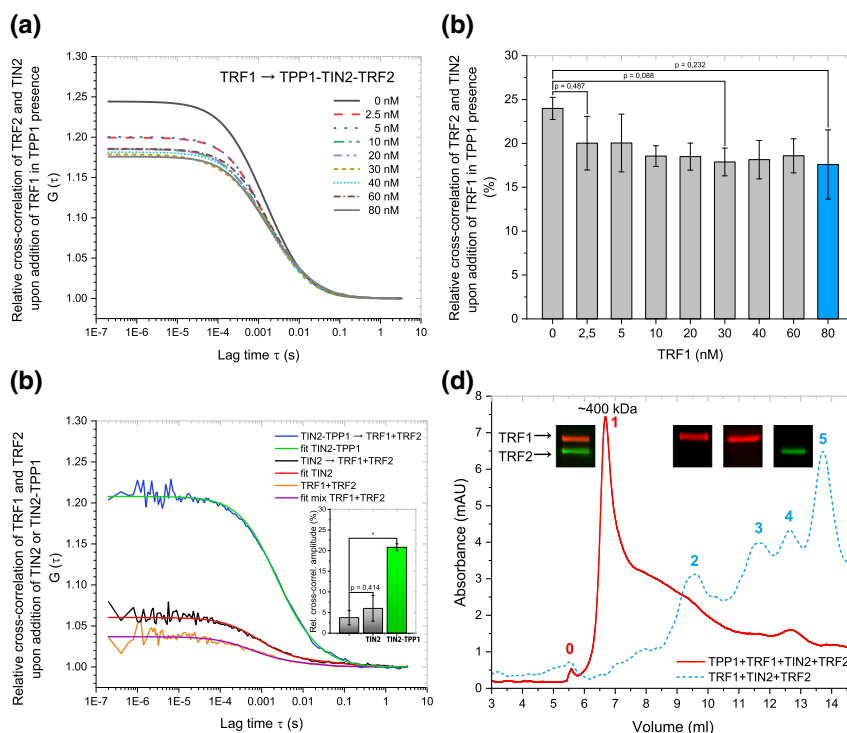


Fig. 4. TPP1 enhances the formation of TRF1-TIN2-TRF2 complex—TRF2 remains bound to TIN2-TPP1 complex in TRF1 presence. (a) Alexa Fluor 488-labeled TIN2 (10 nM) and Alexa Fluor 594-labeled TRF2 (20 nM) were incubated in stoichiometric ratio with unlabeled TPP1 (10 nM) at 25 °C. Fits of relative cross-correlation between TRF2 and TIN2 upon addition of unlabeled TRF1 to TRF2-TIN2-TPP1 complex show significant cross-correlation in TRF1 presence up to 80 nM. (b) The amplitudes of TRF2-TIN2 relative cross-correlation in TPP1 presence upon TRF1 addition—determined from panel a. Error bars represent standard deviations of three independent measurements. *P*-values: two-tailed Student's *t* test with regard to the amplitude without TRF1 (0 nM). (c) Relative cross-correlation of Alexa Fluor 594-labeled TRF1 (20 nM) and Alexa Fluor 488-labeled TRF2 (20 nM) measured upon addition of unlabeled TIN2 (10 nM) or preformed TIN2-TPP1 complex (10 nM). Upon addition of preformed TIN2-TPP1 complex, the significant increase of relative cross-correlation of TRF1 and TRF2 was observed. The TRF1-TIN2-TPP1-TRF2 complex was formed only if TPP1 was present. The inset bar plot shows the increase of TRF1-TRF2 relative cross-correlation upon addition of TIN2-TPP1 complex or TIN2 alone. *P*-values: two-tailed Student's *t* test with regard to the amplitude of TRF1 and TRF2 mixture only; $*P < 0.05$. (d) Size-exclusion chromatography traces of TRF1, TIN2, TPP1, TRF2 (solid line) and TRF1, TIN2, TRF2 (dashed line). TRF1 and TRF2 were labeled by Alexa Fluor 594 and Alexa Fluor 488, respectively. Fractions corresponding to the numbered peaks were collected and analyzed on SDS-PAGE. Peak 0 corresponds to the void fraction. Peak 1 contains both labeled proteins within TRF1-TIN2-TPP1-TRF2 complex. Peak 2 represents TRF1-TIN2; peak 3, TRF1; peak 4, TRF2; peak 5, TIN2. Dynamic light scattering measurements verified TRF1-TIN2-TPP1-TRF2 complex formation (Fig. S9).

Discussion

Our findings of how human telomeric proteins TRF1 and TPP1 affect the formation of the core shelterin complex TRF1-TIN2-TRF2 have provided new insights into the assembly of the full shelterin complex at the single-molecule level. In addition, our study has contributed to address the following biological questions about human shelterin:

- What are the arrangements of shelterin proteins TRF1, TRF2 and TIN2 in solution without DNA?
 - How does the shelterin core TRF1-TIN2-TRF2 assemble?
 - How can TPP1 affect TRF1-TIN2-TRF2 complex formation?
- We address these questions below.

Our quantitative biophysical observations clearly showed that TIN2 binds either TRF1 or TRF2. Thus, two independent complexes TRF1-TIN2 and TRF2-

TIN2 appear in solution. The observation of mutual exclusive binding of TRF1 or TRF2 to TIN2 is in agreement with previous studies. O'Connor *et al.* [42] have suggested that TRF1–TIN2 and TRF2–TIN2 occur as separate sub-complexes based on immunoprecipitation studies. In addition, when we take into consideration available information about the positions of interacting domains, domain structure and quantitative binding characterizations, we can rationalize why TRF1 replaces TRF2 on TIN2. So far, it seemed that the shelterin proteins form hetero-multimeric complexes through a selective domain-domain interaction mechanism.

As Chen *et al.* [18] showed, both TRF2 and TRF1 can bind one common binding site TBM (TRFH-binding motif Fig. 1a) on TIN2. TBM at the C-terminus of TIN2 is a well-structured 256–276 region that interacts with TRFH domain of both TRF1 and TRF2. The surface of TBM matches better the hydrophobic interface of TRFH domain of TRF1 than the polar interface of TRFH domain of TRF2. The different structural arrangements of interaction interfaces prompt the TRFH domain of TRF1 to bind a peptide representing the TBM region of TIN2 with higher binding affinity than the TRFH domain of TRF2 [18].

In addition, there is another well-structured binding site at TIN2's N-terminus (TRFH-like) where TRF2 binds with higher affinity compared to the previously mentioned common TRF1/TRF2 binding site TBM. If we consider that interactions between proteins occur mainly through the minimal identified domains, we may expect similar binding affinity for full-length proteins. Thus, TRF1 may form a complex with TIN2 more readily than TRF2. The higher binding affinity of TRF1 to TIN2 causes higher preference for the formation of the complex TRF1–TIN2 compared to TRF2–TIN2.

We determined the ensemble binding affinity of full-length TRF1 to TIN2 and TRF2 to TIN2 by microscale thermophoresis (Supplementary Fig. S5). Here, the obtained binding affinities for full-length proteins are higher than the affinities for isolated domains measured by Chen *et al.* [18] and Hu *et al.* [19]. The elevated binding affinity might suggest that additional hydrophobic and hydration effects promote full-length protein interactions [45].

If we consider the higher binding affinity of TRF1 to TIN2, we suppose that the TRF1–TIN2 complex incidence should prevail over the TRF2–TIN2 complex occurrence. In addition, if there was no other binding site on TIN2 for TRF2 or a binding regulation mechanism, the probability of forming complex TRF1–TIN2–TRF2 would be significantly low. The second binding site for TRF2 on TIN2 (TRFH-like domain, Fig. 1a) should allow the formation of the trifunctional complex TRF1–TIN2–TRF2.

In this context, our finding that TRF1 can substitute TRF2 when bound to TIN2 was rather unexpected at

first glance (Fig. 2a and b). However, we can rationalize the TRF2 displacement when we consider that TRF1 binding affinity to TIN2 is higher [19] than TRF2 binding to TBM on TIN2 [18], as supported by our microscale thermophoresis measurements with full-length proteins (Supplementary Fig. S5). Let us assume that TRF2 binds TRFH-like domain of TIN2 in TRF1 absence, as TRF2 should occupy the binding site with the highest affinity at first. When TRF1 appears in TIN2–TRF2 complex proximity, TRF1 binds TBM site on TIN2.

We observed that the relative cross-correlation between TRF2 and TIN2 decreased even before the equal concentration of TRF1 and TRF2 was reached (Fig. 2a). We may hypothesize that (i) TRF1 binding to TIN2 affects TRF2 release catalytically; (ii) TRF1 simultaneously binds several TRF2–TIN2 subunits; and (iii) TRF2 forms aggregated clusters that are released from TIN2 by TRF1 before the expected equimolar ratio TRF1:TRF2 is achieved. The last explanation seems to be the most probable regarding our observation that TRF2 aggregates on DNA (Fig. S6, Video 1). Unfortunately, based on the available data, we could not determine the main cause of the premature drop of relative cross-correlation.

Why is TRF2 released upon TRF1 binding when there are two independent binding sites for TRF1 and TRF2? One straightforward explanation would be that TRF1 bound to TIN2 presents a steric hindrance that disturbs the optimal interaction surface between TRF2 and TIN2. The second explanation could be that TRF1 induces structural changes in TRFH-like domain of TIN2 that disable TRF2 binding to TIN2. Moreover, there could be a combination of both—steric hindrance and structural changes. Nevertheless, TRF2 binding to TRF1–TIN2 must be promoted to form the stable core complex TRF1–TIN2–TRF2.

As the first, O'Connor *et al.* [42] suggested that TPP1 promotes the interaction between TIN2 and TRF2. Recently, Hu *et al.* have shown that the binding of TPP1 interacting domain to TIN2 allosterically changes the TRF2 binding site on TIN2 TRFH-like domain [19]. Furthermore, this study revealed that the binding affinity between minimal interaction domains of TPP1–TIN2 and TRF2 was increased almost 3-fold if compared to the interaction without TPP1 [19].

As TPP1 induces allosteric changes on TIN2 that open the second binding site and thus increase TRF2 binding affinity, TPP1 promotes the stable formation of TRF1–TIN2–TPP1–TRF2 complex. Our single-molecule FCCS results support the view that TPP1 acts as a shelterin assembly activator. We corroborated that TRF2 remained bound to TIN2 in TRF1 presence and TRF1–TIN2–TRF2 complex was formed when TPP1 was bound to TIN2 (Fig. 4a and c). We summarize our recent results within the framework of the present state of knowledge of shelterin core assembly in the model below.

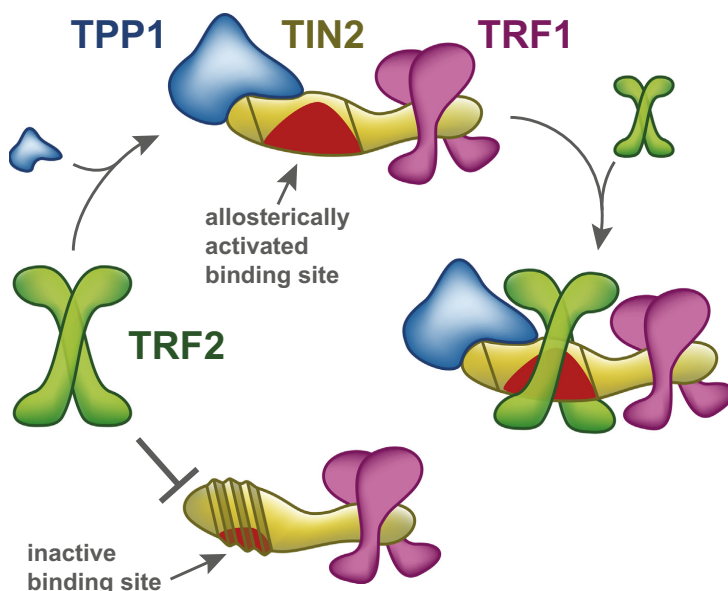


Fig. 5. A model for sequential assembly of shelterin core complex TRF1–TIN2–TRF2. TRF1 prevents TRF2 from binding to TIN2 if TPP1 is absent, as TRF1 occupies the preferential binding site on TIN2 (lower part of the scheme). On the contrary, when TPP1 binds TIN2, TPP1 induces structural changes that open the second binding site on TIN2, and the binding site for TRF2 becomes active (upper part of the scheme). TRF2 binds TIN2–TPP1 along with TRF1 (middle part of the scheme). A stable shelterin core complex TRF1–TIN2–TPP1–TRF2 is formed.

Model of core shelterin assembly

We propose a model of how shelterin core proteins assemble in solution. The model takes into consideration that TRF1 and TRF2 form homodimers [46,47], as the homodimerization exclusivity of TRF1 and TRF2 is a functional requirement that facilitates separation of different domain functions for both TRF proteins of similar domain structures. The model also reflects the stoichiometry of shelterin proteins that has been revealed by the de Lange and the Cech laboratories [34,35].

The recommended model suggests that if TRF1 occupies the TBM binding site on TIN2, TRF2 binding to TIN2 is compromised—only TRF1 remains bound to TIN2 (Fig. 5). When TPP1 binds TIN2, the N-terminal binding site of TIN2 becomes active. Subsequently, TRF2 can bind TIN2 also in TRF1 presence. Thus, TPP1 activates the N-terminal binding site for TRF2 on TIN2 and enables TIN2 to accommodate both TRF1 and TRF2 simultaneously. The model suggests that the protein order during shelterin core self-assembly in solution is TRF1 > TIN2 > TPP1 > TRF2.

In addition, the model explains the unique ability of TRF1 to exclude TRF2 from the complex TRF2–

TIN2. Our model suggests that TRF1–TIN2 is an initial complex based on the highest affinity between TIN2 and TRF1 among shelterin proteins [18]. Moreover, TRF1–TIN2 preferential binding explains why it is not possible to prepare TRF1–TIN2–TRF2 complex without TPP1. The model is in agreement with recent findings that suggest that TPP1 induces allosteric structural changes on TIN2 to open the N-terminal TRF2 binding site [19]. As Hu *et al.* showed by fluorescence polarization measurements, TPP1 upon binding to TIN2 increases the affinity of the interacting domains of TRF2 to TIN2 [19]. Accordingly, Kim *et al.* [48] used isothermal titration calorimetry to detect 17-fold increase in binding affinity of TRF2 and TIN2 upon complexation with TPP1. Thus, the induced structural changes allow tighter binding of TRF2 to TIN2 without the compromising effect of the prebound TRF1.

The model with two TRF2 binding sites on TIN2 has been supported by our FCCS experiments with mutated variants of TIN2 (Supplementary Fig. S4). We observed no cross-correlation for TIN2 mutants that were unable to bind TPP1 or TRF2 on the N-terminus of TIN2. The point mutations of TIN2 prevented the complexation of TRF1 and TRF2. The model supports the view that the N-terminal

binding domain of TIN2 is essential for the cooperative binding of TRF2 and TPP1 to TIN2 and promoting assembly of TRF1–TIN2–TRF2–TPP1 complex.

In addition, we carried out FCCS experiments with monomeric TRF2V_{52D,N53P}—the construct with impaired self-dimerization [49]. We observed that the relative cross-correlation between fluorescently labeled TRF2V_{52D,N53P} and fluorescently labeled TIN2 was diminished upon unlabeled TRF1 addition (Supplementary Fig. S10a and b). In other words, TRF1 released monomeric TRF2V_{52D,N53P} from TIN2. Furthermore, we have carried out the control titration of TRF1 to TRF2V_{52D,N53P}–TIN2–TPP1 (Supplementary Fig. S10c and d). The comparison of the control titration with the experiment when TRF1 was titrated into TRF2V_{52D,N53P}–TIN2 (Supplementary Fig. S10a and b) shows that TRF1 releases TRF2 also when TRF2 is in the monomeric form. The lower deviation of relative cross-correlation amplitudes observed when TRF1 was titrated into monomeric mutant TRF2 containing complex TRF2V_{52D,N53P}–TIN2–TPP1 (Fig. S10c) compared to the deviation of relative cross-correlation amplitudes when TRF1 was titrated into wild-type TRF2–TIN2–TPP1 (Fig. 4a) might be because the monomeric TRF2V_{52D,N53P} prevents nonspecific interactions with TIN2. We observed that TRF1 induced the release of monomeric TRF2V_{52D,N53P} from TIN2 in similar extent as the release of wild-type TRF2 from TIN2 (Fig. 2a and b), which further supports the suggested binding mechanism. Thus, the proposed model is robust that it may be extended to TRF2 that binds to TIN2 as a monomer.

The proposed model is applicable also to mechanisms where proteins first form weak transient complexes, and then depend on the additive energies of binding and structural changes provided by partner proteins to generate higher specificity. Finally, it should be pointed out that the intrinsic dynamics of TRF1 and TRF2 could be important for regulating the assembly and disassembly of shelterin complexes, and exchanging between capped and uncapped telomere structures [50]. Based on here documented single-molecule studies, we suggest that TIN2–TPP1 binding could function as a switch to allow complete shelterin assembly containing TRF1 that associates with double-stranded DNA and TRF2 that associates mainly with the double-strand/single-strand junction of telomeric DNA. Thus, TPP1 binding seems to be critical for the whole shelterin assembly. Then the complete shelterin could accumulate on telomeres properly and maintain its regulatory functions regarding telomerase access and activity. Recently, we revealed that the interaction of TIN2 and TPP1 shows the lowest affinity within shelterin subunits *in vitro* [51]. Thus, TPP1–TIN2 interaction is a limiting step during shelterin reconstitution. Hence, our results

along with recent functional and structural studies advocate that TPP1–TIN2 interaction is crucial for both—functional shelterin assembly and dynamics of shelterin reconstitution.

In summary, our study with full-length human telomeric proteins TRF1, TRF2 and TIN2 brings new information about the assembly of shelterin sub-complexes. Our results with the core shelterin complex TRF1–TIN2–TRF2 extend the knowledge, so far limited to previous structural and functional studies of shelterin assembly that have been carried out without TRF1. For the first time, we applied single-molecule approaches to monitor TRF1, TRF2, TIN2 and TPP1 during their assembly in solution. The presented studies describe how the mutual arrangement of functional subcomplexes of telomeric proteins contribute to the role of the whole shelterin in telomere protection. The next challenging tasks will be to monitor the dynamics of the shelterin complex assembly in living cells.

Material and Methods

Cloning, expression and purification of TRF1, TRF2, TIN2 and TPP1

The cDNA sequences of TRF1, TRF2, TIN2 and TPP1 were synthesized by Source BioScience and cloned to pDONR/Zeo vector (Life Technologies) using two sets of primers and BP clonase enzyme mix from Gateway technology (Life Technologies). The resulting plasmids were cloned into different expression vectors in a recombination reaction using LR clonase enzyme mix (Life Technologies) and expressed as His-tagged proteins in different strains of *Escherichia coli* (pUbiKan_X105_TRF1 and pHGWA_TRF2 in BL21(DE3), pTriEx4_TIN2 in C41 and pRbXKan_x105_TPP1-FL (89–554) in BL21 (DE3) RIPL. BL21(DE3) and C41 cells harboring TRF1, TRF2 and TIN2 were grown in Luria-Bertani medium, and BL21(DE3) RIPL cells with TPP1 were grown in Terrific Broth medium, containing 50 $\mu\text{g ml}^{-1}$ kanamycin (TRF1, TPP1) or 100 $\mu\text{g ml}^{-1}$ ampicillin (TRF2, TIN2) or 34 $\mu\text{g ml}^{-1}$ chloramphenicol (TPP1) at 37 °C until A_{600} reached 0.5 (TPP1) or 1.0 (TRF1, TRF2, TIN2). The cells were cultured for 3 h at 15 °C (TRF1, TPP1) or 25 °C (TRF2, TIN2) after the addition of IPTG to the final concentration of 0.5 mM (TRF1, TIN2, TPP1) or 1 mM (TRF2). Cells were collected by centrifugation (8000g, 8 min, 4 °C).

The pellet was dissolved in lysis buffer containing 50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 10 mM imidazole (TRF1, TRF2, TIN2) or 20 mM imidazole (TPP1), 0.5% Tween-20 (TRF1, TRF2, TIN2) or 0.5% Triton X-100 (TPP1), 10% glycerol, and protease inhibitor cocktail cComplete tablets EDTA-free (Roche). The cell suspension was

sonicated for 3 min of process time with 1-s pulse and 2 s of cooling on ice (Misonix). Cell lysate supernatant was collected after centrifugation at 20,000g, 4 °C for 1 h. Proteins were purified by immobilized-metal affinity chromatography using TALON® metal affinity resin (Clontech), where filtered supernatant (0.45 µm filter) was mixed with TALON® beads and incubated for 30 min. The proteins of our interest were eluted at 200 mM (TIN2), 300 mM (TPP1) or 500 mM (TRF1, TRF2) imidazole in the same buffer without Tween-20 or Triton X-100. TRF2 and TIN2 were dialyzed into 50 mM sodium phosphate (pH 7.0) and 50 mM NaCl. TRF1 was loaded onto the HiLoad 16/600 column containing Superdex 200 pg (GE Healthcare Life Sciences) and resolved using 50 mM sodium phosphate buffer (pH 7.0) with 200 mM NaCl. TRF1 was expressed and purified with tags His, S and Ubi to extend the protein stability during FCCS experiments at 25 °C. Tags on TRF1 did not affect FCCS measurements, as documented in Supplementary Fig. S11. TPP1 expression tags were removed by HRV3-C protease at 4 °C for 2 h with 3 mM DTT. The final purification was on HiLoad Superdex 200 pg column (GE Healthcare Life Sciences) equilibrated with buffer containing 50 mM sodium phosphate (pH 7.0) and 800 mM NaCl. The proteins were concentrated and the buffer was exchanged to 50 mM sodium phosphate (pH 7.0), 50 mM NaCl (TRF1, TRF2, TIN2) or 150 mM NaCl (TPP1) by ultrafiltration (Amicon 3 K/30 K, Millipore).

The concentration of purified proteins was determined by the Bradford assay. We evaluated protein purity by SDS-polyacrylamide gels stained by Bio-Safe Coomassie G250 (Bio-Rad). Western blotting and quantitative mass spectrometry analyses also confirmed the presence of proteins.

DNA substrates

For the DNA binding affinity studies, DNA duplex R5 was prepared by annealing a fluorescently labeled oligonucleotide (Alexa Fluor 488) with the sequence 5'-GTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGT-TAG-3' and its complementary strand. The sequence of R5 was designed in accordance with the optimal binding site of TRF2 defined by the de Lange laboratory [52]. The substrate was purified using a Mono Q 5/50 GL column (GE Healthcare) with a gradient of 50–1000 mM LiCl in 25 mM Tris-HCl (pH 7.5). All oligonucleotides were purchased from Sigma-Aldrich.

Fluorescence anisotropy

Measurements of TRF1–TIN2 and TRF2–TIN2 binding to telomeric DNA duplex R5 labeled by Alexa Fluor 488 were performed on a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ). Fluorescence anisotropy was monitored at an

excitation wavelength of 490 nm and emission wavelength of 520 nm. The slit width (both excitation and emission) for all measurements was 9 nm and the integration time was 1 s. The cuvette contained 1.4 ml of DNA duplex R5 (7.5 nM) in a buffer containing 50 mM sodium phosphate (pH 7.0) and 50 mM NaCl. The protein mixture was titrated into the DNA solution in the cuvette and measured after a 2-min incubation at 25 °C. Fluorescence anisotropy at each titration step was measured five times and averaged with relative standard deviation always lower than 3%. The values of dissociation constants were determined by non-linear least square fits according to the equation $r = \frac{r^{MAX}}{1 + (K_D + c)}$ using ORIGIN® 2018 (OriginLab, Northampton, MA) and confirmed by symbolic equation-based fitting using Dynafit [53].

Fluorescent protein labeling

Fluorescent protein labeling was performed according to the protocol provided by the supplier with the following modifications. Alexa Fluor 488, carboxylic acid, 2,3,5,6-tetrafluorophenyl ester or Alexa Fluor 594 carboxylic acid, succinimidyl ester (Molecular Probes–Invitrogen) in 4-fold molar excess over protein were diluted in 1/10 protein volume of 1 M sodium bicarbonate, fluorophores were mixed with protein (1 mg) and incubated for 1 h at 4 °C while stirring. The mixture was loaded on PD-10 columns (GE Healthcare) and eluted with 50 mM sodium phosphate (pH 7.0) and 50 mM NaCl. The degrees of labeling—dye/protein ratio—were 95% for TRF1, 93% for TRF2 and 97% for TIN2, as determined by UV/Vis spectroscopy.

Theoretical concept of FCS and FCCS

Fluorescence correlation spectroscopy (FCS) describes spontaneous fluorescence intensity fluctuations caused by rapidly diffusing molecules in a microscopic detection volume (about one femtoliter). FCS determines mobility and kinetics at single-molecule precision [39]. FCCS monitors two different fluorescence signals (two colors) collected at the same time and determines how their coincident fluctuations correlate to each other if the proteins are moving together. FCCS describes binding of measured proteins independently of diffusion rate. The cross-correlation function of two-color system, with one green-labeled particle *G* and the second with red-labeled particle *R*, is described as follows

$$G_{GR}(\tau) = \frac{\langle \delta F_G(t) \cdot \delta F_R(t + \tau) \rangle}{\langle F_G(t) \cdot F_R(t) \rangle} \quad (1)$$

where $F_G(t)$ and $F_R(t)$ correspond to fluorescence intensity fluctuations of individual signals (green and red) and τ represents lag time—the time period that

two proteins stay in confocal volume together. A typical cross-correlation curve is a sigmoidal curve with the highest amplitude in time $t(0)$. With longer lag time, the amplitude decreases, as the probability that two proteins are bound and stay in the confocal volume is lower with increasing time. The single-molecule nature of FCCS measurements causes that relative cross-correlation amplitudes of the same sample fluctuate within standard error, because different number of molecules is detected in confocal volume at different times. The cross-correlation fit with an appropriate model provides us with the degree of complexation of two fluorescently labeled molecules. Under ideal conditions (absence of fluorescence resonance energy transfer and spectral crosstalk of used fluorophores), the cross-correlation amplitude $G_{GR}(0)$ is directly proportional to the concentration of bound species [39,41].

Relative cross-correlation as a measure of binding

In FCCS experiments, we used TIN2 labeled with Alexa Fluor 488 and TRF1 or TRF2 labeled with Alexa Fluor 594, if not stated otherwise. For data evaluation, we refer to relative cross-correlation calculated as a ratio between amplitude of cross-correlation function $G_{TIN2-TRF}(0)$ and auto-correlation function $G_{TIN2}(0)$. In other words, the relative cross-correlation corresponds to the proportion of cross-correlation amplitude related to the TIN2 auto-correlation amplitude. This approach also allowed us to normalize the data regarding their concentration so we could directly compare the results of all measured FCCS experiments [39].

Conditions for microscopy imaging and spectroscopy measurements

All the FCCS measurements were performed with a confocal laser scanning microscope Zeiss LSM 780 using dedicated software ZEN Studio with additional FCS module. For excitation, the Ar⁺ laser was used with 488- and 561-nm continuous wave. The emission filters were tuned to minimize crosstalk between Alexa Fluor 488 and Alexa Fluor 594 [54]. To determine the crosstalk, we carried out a FCCS measurement of a mixture of free fluorophores Alexa Fluor 488 and Alexa Fluor 594 that served also as a negative control with minimal cross-correlation (Supplementary Fig. S1). To determine the maximal cross-correlation, we used DNA duplex with two fluorophores attached to opposite ends as a positive control. The positive control consisted of Cy5 and Alexa Fluor 488-labeled 40-mer oligonucleotide with the sequence 5'-[Cy5]-TACTAGTTCACCGTCAGATCCACTAGCAGCTAG-TTCGAT-[Alexa488]-3' that was hybridized with its complementary strand. The positive control was

measured under the same conditions as the negative control. Confocal pinhole was fixed to 1 AU, and it was additionally fine adjusted in x , y directions prior to FCCS measurement to maximize the count rate of fluorescence fluctuations. We used "FCS approved" water immersion objective (Zeiss 63x C-Apochromat NA 1.2 W Corr), which guarantees overlap of the point spread function for different wavelengths and correction for any spherical aberration.

Fluorescently labeled TIN2 and TRF1 or TRF2 were incubated at 25 °C at 1 μ M concentration to form a complex. The sample was diluted to the final concentration that guaranteed the optimal amount of labeled proteins diffusing through the confocal volume at the same time (up to five proteins) corresponding to concentration 10–20 nM.

Prepared samples were incubated at room temperature, diluted with 50 mM sodium phosphate buffer (pH 7.0) with 50 mM NaCl to a final volume of 200 μ l and loaded into a μ -slide 8-well Glass Bottom Chamber (Ibidi). The sample covered the entire bottom of the well. Measurements were started immediately, without further incubation.

For each experiment, raw data containing 50 repetitions of 10-s acquisition were collected and averaged. This approach ensured that we collected enough data to obtain statistically significant values. The raw data were exported and analyzed with software QuickFit 3 [55]. Correlation curves were averaged and fitted with two-component 3D Normal Diffusion model by solving the Levenberg–Marquardt nonlinear least-squares fitting routine. The data were visualized by ORIGIN® 2018 (OriginLab, Northampton, MA). The experiments were performed in triplicate. P -values were calculated using two-tailed Student's t test by Statistica 13 (Dell).

Size-exclusion chromatography

Protein samples for size-exclusion chromatography were centrifuged and filtered through 0.22- μ m membrane filters to avoid aggregations. TRF1, TIN2, TPP1 and TRF2 were mixed and incubated in the same order as they appear. The protein molar ratios were preserved in accordance with the FCCS measurements (TRF1/TRF2/TIN2/TPP1 2:2:1:1). TRF1 and TRF2 were labeled by Alexa Fluor 594 and Alexa Fluor 488, respectively. Superdex™ 10/300 GL column with 50 mM NaCl and 50 mM phosphate (pH 7.0) as mobile phase and flow rate 0.5 ml/min was used for the chromatographic separation. Fractions corresponding to the numbered peaks were collected and analyzed on SDS-PAGE. Labeled proteins in gels were detected using Typhoon™ FLA 9500 detection system. Collected fractions were characterized by Dynamic Light Scattering (Fig. S9) to verify TRF1–TIN2–TRF2–TPP1 complex formation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmb.2019.05.038>.

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TRF, telomere repeat-binding factor; TIN2, TRF1-interacting nuclear factor 2; POT1, protection of telomeres 1; FCCS, fluorescence cross-correlation spectroscopy; FCS, fluorescence correlation spectroscopy.

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Research article 2

Enhancement of luminescence signal by deuterated water – Practical implications

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Enhancement of luminescence signal by deuterated water – Practical implications

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ABSTRACT

The fluorescence signal increase in deuterated water - D₂O is relevant to many methods used in chemical and biological research. We performed fluorescence spectroscopy measurements combined with evaluations of fluorescence-based methods to describe and quantify the practical impacts of D₂O on chemical and biological samples. Several novel features are observed in fluorescence spectra and life-time decay curves of molecules including fluorescence probes and labels that are used frequently in biomolecular labelling and bioimaging. The observed characteristics suggest that D₂O enhances the fluorescence signal due to the exchange of labile hydrogen on a fluorophore for heavier deuterium and the reduction of energy transfer from excited fluorophores to H₂O molecules. Furthermore, performed quantifications prove that D₂O increases significantly the detection signal of all examined fluorescence-based approaches where the brightness of fluorophores and sample viability are crucial.

1. Introduction

Fluorescence (luminescence) detection is widely used in analytical instrumentations for qualitative and quantitative analyses of many organic and inorganic molecules [1,2]. Fluorescence detections is often employed in techniques essential for medicinal and biological research such as flow cytometry, fluorescence microscopy, fluorescence lifetime imaging microscopy (FLIM), and fluorescence correlation spectroscopy (FCS) with light water as a medium in a majority of luminescence experiments. As fluorescence is extremely dependent on the molecular environment (solvent, pH, ionic strength, etc.), the choice of light water medium often results in lower fluorescence intensities due to unwanted quenching when compared to other solvents [1]. Importantly, a simple substitution of H₂O to D₂O significantly reduces fluorescence quenching and thus increase the fluorescence quantum yield observable even by the naked eye (Fig. 1). An increase higher than 100% (ratio of fluorescence intensities in both solvents, $I_D/I_H > 2$) would be attractive from an analytical point of view – it enables reaching lower limits of detection

(LOD) or decreasing concentrations of the used dye. Although the primary usage of heavy water is found in nuclear power plants, pure deuterated water is not radioactive, and its toxicity is relatively low [3]. It is also commercially available with the price being around 1 USD per 1 ml of 99% D₂O [4].

The first published description of the enhancement of luminescence by deuterium atom has been made by Hutchison et al. [5] At the same time, Robinson [6] described luminescence lifetimes of deuterated benzene at low temperatures and suggested that heavier atoms of deuterium show smaller vibrational overlap than protium resulting in a smaller energy transfer from the excited molecule. Stryer observed in 1966 that some aromatic molecules exhibit higher fluorescence intensities when put in deuterated water [7]. He noticed that only molecules containing a proton-donor group were able to increase their fluorescence intensity in deuterated water. He rationalised the effect as a different rate of a proton or deuterium transfer in the excited state. A year later, T. Förster and K. Rokos demonstrated that the isotopic effect of the solvent is involved as well [8]. Consequently, J. Eisinger, G. Navon, and

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Fig. 1. Photography of (from left to right) fluorescein (pH (pD) = 2), doxorubicin, rhin, ethidium bromide, propidium iodide (pH(pD) = 7) in H₂O (left) and D₂O (right) irradiated by violet light. The difference in fluorescence intensity of each couple can be easily observed by the naked eye.

R. W. Ricci published works concerning fluorescence quenching and isotope effect in the indole and its derivatives [9,10]. An increase in fluorescence intensities has been observed for an NADH molecule and its complexes by Blomquist [11], as well as in an intercalation study of several organic dyes with specific DNA structures [12].

Recently, Lee et al. used deuterated water to improve resolution in fluorescence microscopy using red-fluorescent oxazine dyes in various solutions [13]. They noticed that fluorescence efficiency decreases with the increasing ability of the solvent to form hydrogen bonds. Another fluorescence microscopy experiment in D₂O with cyanine dyes performed by Lee et al. addressed the differences in fluorescence emission spectra to the higher absorption rate of water molecules in the visible region of light [14]. A few examples of molecules able to benefit from the use of deuterated water in chromatography with fluorescence detection [15], and spectrometric determination of deuterated water in the presence of light water have been demonstrated by our group [16, 17].

2. Material and methods

Experimental details and materials are provided in [Supplementary Information](#) file.

3. Results and discussion

We quantified luminescence yields and lifetimes changes upon substituting the light water for deuterated water. We have assumed the alternation of luminescence properties of molecules based on the solvent should be introduced with related examples and experiments. It is worth noting that the luminescence enhancement may be attributed to multiple phenomena as molecules in solutions may strongly interact with each other. However, the major luminescence enhancing contributions might be addressed to the following processes: deuterium isotope exchange effect, reduction of excited-state proton transfer (rESPT), energy transfer (ET), hydrogen bond assisted quenching, and reduction of tautomerisation (ESIPT). Each of these will be presented and discussed in the text.

3.1. Rapid exchange of protons for deuterons in the molecule structure

Rapid exchange of protons originating from labile hydrogens (typically, hydrogens from –OH, or –NH_n groups) for deuterons occurs in a D₂O solution immediately after mixing at room temperature [18]. Experiments with cresyl violet in a gaseous state, in which the solvent effects are entirely suppressed, confirmed that the exchange of four protons for deuterons caused a 17% and 19% increase in luminescence lifetime and intensity, respectively [19]. The presence of labile hydrogens allowing the proton/deuteron exchange affected the rates of non-radiative processes. Indirect evidence of the exchange process contributing to altering luminescence properties was observed by our

group on fluorescence characteristics of fluorescein across a wide range of pH. While at a high pH (~12) the completely deprotonated fluorescein molecule (pK_a ~6.4) showed almost identical fluorescence lifetimes regardless of the solvent, any lower pH kept enlarging the lifetime difference in D₂O (Fig. 2a, b). The proton/deuteron exchange substantially increases the luminescence quantum yield of DNA probes – propidium iodide (Fig. 2c, d) and ethidium bromide [12]. The luminescence intensity is clearly dependent on D₂O concentration (Fig. 2e). Both molecules probably relax energy through an N-H stretching mode. High frequency of N-H stretching supports S₁→S₀ non-radiative internal conversion. The lower stretching frequency of N-D causes retardation of the non-radiative process enabling the emission of a photon. Analogously, tryptophan and other indol derivatives undergo the enhancement (50–150%) at different pH in D₂O [9,10]. The experiments with DMSO-d₆ and DMSO have shown that labile hydrogen exchange is suppressed in aprotic solvents and the difference in fluorescence intensity was recored to be only 2% in case of ethidium bromide (Fig. S14).

Other examples in the literature suggest that even non-labile hydrogens (typically in C-H bonds) might be substituted for deuterons during complex synthetic reactions. In general, such non-labile deuterium atoms should also reduce internal conversion and thus enhance the luminescence quantum yield. Experiments with azulene-d₈ and azulene-h₈ showed a 40% increase of quantum yield in the case of a perdeuterated molecule [20]. Incorporation of non-labile deuterium atoms into pendant parts of rhodamine derivatives significantly improved fluorescence quantum yields [21]. Nevertheless, in literature are described also rarely observed inverse effect (decrease of luminescence after deuteration) [20].

As any labile hydrogens might be substituted by deuterium atoms at room temperature after the addition of D₂O (or deuterated alcohols), then molecules having a large number of labile hydrogens are anticipated to increase both fluorescence intensity and lifetime (up to 200%) in an effortless way of changing a solvent. The emission maxima in deuterated solvents are not significantly shifted (Fig. 2c), and the total number of exchanged hydrogens can be viewed, e.g., by mass spectrometry (Fig. 2d, more MS spectra in supplementary). As molar extinction coefficients are usually almost identical in both solvents; the I_D/I_H is then similar to a quantum yield ratio (φ_D/φ_H). When the molecule is present in a deprotonated form, the I_D/I_H and τ_D/τ_H ratios are close to one (Fig. 2a).

3.2. Reduction of the excited-state proton transfer

L. Stryer et al. suggested a scheme (Scheme 1) describing the excited-state proton transfer (ESPT) to water as an explanation of fluorescence enhancement of emission in D₂O [7]. The R-XH (resp. R-XD) is a protonated form of the fluorescent molecule, and *R-XH represents the molecule in the excited S₁ state. The excited molecule can emit a photon or can undergo a reaction with H₂O (respectively D₂O) to form a deprotonated form (*R-X⁻). The rate of the emissive process is represented by k_F, whereas the rate constants of formation of *R-X⁻ is k₁ and the rate constant of dissociation is k₂. The non-radiative de-excitation process is then expressed by k_T and k_T constants. The rate of formation of the deprotonated form is usually faster in H₂O than in D₂O due to the slower deuterium transport [7,8,12]. For example, k₁ of 2-naphthol is 5.29 × 10⁷ and 1.31 × 10⁷ in H₂O and D₂O, respectively [7]. In other words, the period for the radiative event from *R-XH state is significantly longer in D₂O than in H₂O. The deprotonated *R-X⁻ forms of molecules can be non-fluorescent (phenol) or fluorescent (2-naphthol) with different emission maximum [7,22]. While the *R-X⁻ form is considerably fluorescent, the overall emission spectrum in H₂O and D₂O differs [22]. But our measurements have revealed only a few examples of significantly different shape of emission spectra in D₂O and H₂O. As an example can be shown emission spectrum of anthraquinone rhin in H₂O with different spectral shape than the sample in D₂O (Fig. 3a)

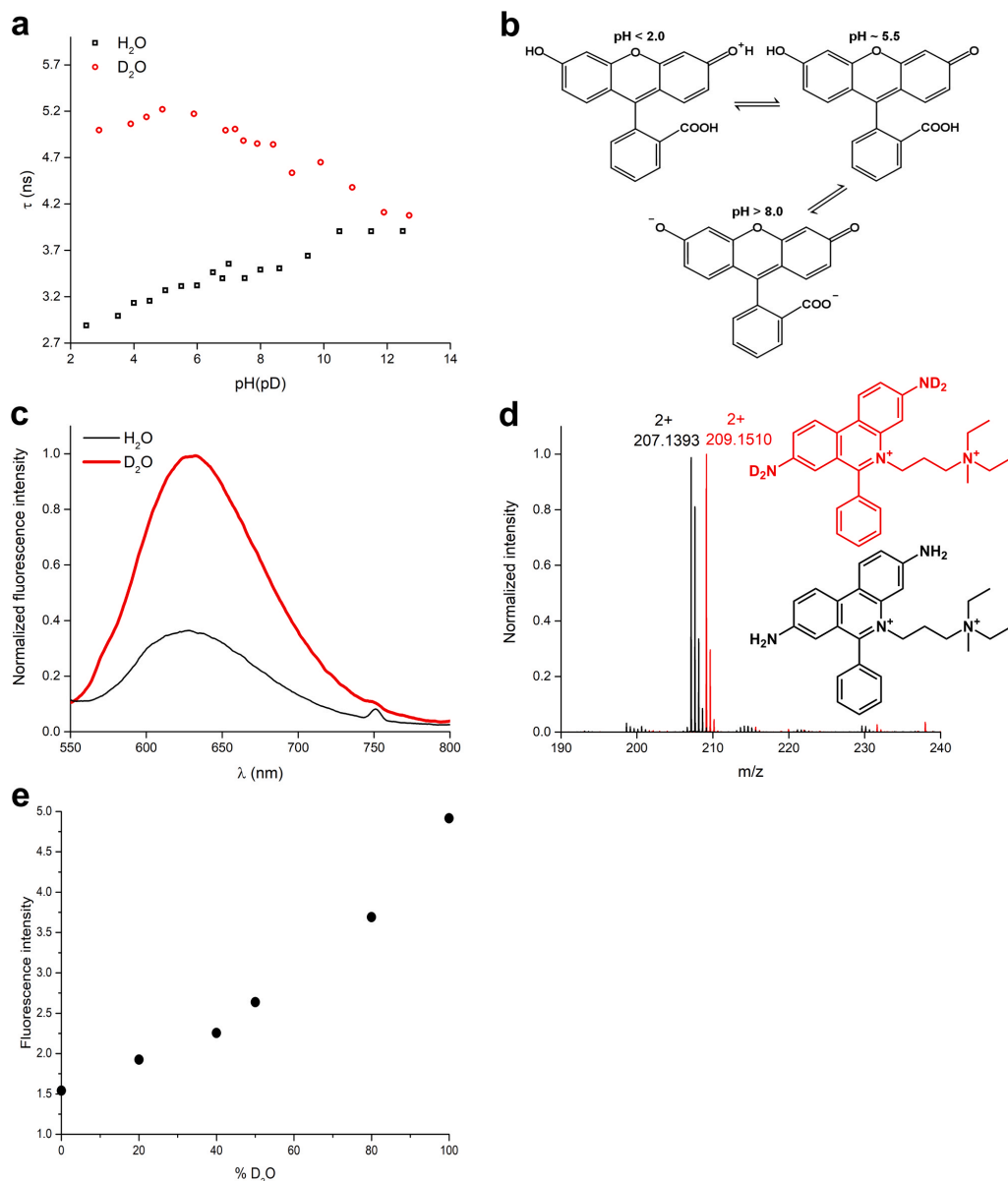
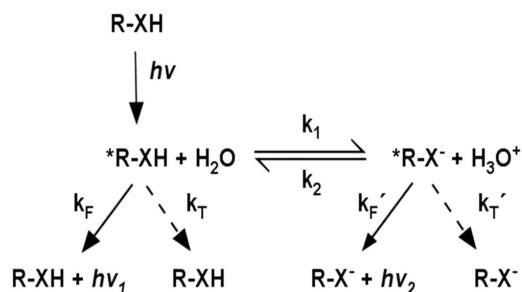


Fig. 2. (a) Dependence of luminescence lifetimes of fluorescein on pH in H_2O (black $\square$) and D_2O (red $\circ$) with the simplified scheme (b) of protonation/deprotonation of the fluorescein molecule. Note similar luminescence lifetimes at higher pH(pD) values indicating the absence of labile hydrogens (deuterons). (c, d) Fluorescence (c) and mass (d) spectra of propidium iodide in D_2O (red line) and H_2O (black line) and its expected structure in both solvents. Mass spectrometry experiment showed an increase in molecular mass indicating the labile hydrogen exchange for deuterons. (e) The dependence of the fluorescence emission on the D_2O concentration of propidium iodide ($\lambda_{em} = 628$ nm, pH(pD) = 7.00).

indicating probably different content of protonated/deprotonated forms in both media.

Among ESPT to solvent can molecules also exhibit the excited-state intramolecular proton transfer (ESIPT) in which the hydrogen bond is formed between hydrogen donor group ($-OH$ or $-NH_2$) and an acceptor

group ($=N-$ or $C=O$) of the same molecule [23–25]. These molecules can (in the excited state) relax down part of their energy through a transfer of proton, which results in tautomerisation, e.g., keto-enol tautomerisation. Both tautomers use to have utterly different emission maxima and tautomer with transferred proton has notably higher



Scheme 1. Expected scheme of ESPT summarising possible non-radiative and radiative de-excitation pathways of a molecule in the excited state. Radiative process (represented by k_F) competes with the photochemical transformation (k_1) and non-radiative process (k_T) [7].

emission maximum [26].

We wanted to verify the theory that ESPT mechanism can be reduced by the presence of D_2O . For our experiments, we selected alizarine red S dye because this molecule can undergo ESPT as was

described in the literature [27]. But our experiments have not shown any significant change in the shape of emission maxima (Fig. 3b). This observation indicates that the ESPT mechanism is not influenced by the presence of D_2O and content of tautomer with a transferred proton is probably very low in both media. Note that undesirable differences in spectral shape (among the fluorescence intensity, see supplementary) can be also achieved by inaccurate pH adjustment (or unbuffered solutions) which can lead to differently protonated species, especially the molecules with pKa values around 7.

3.3. Energy transfer and hydrogen bond assisted quenching

While there is an abundant amount of compounds able to increase its luminescence intensity and lifetime in deuterated water significantly, the mechanisms described above may not elucidate such behaviour. For instance, many commercially available dyes and some other molecules, such as Cy3, Cy3B, Cy3.5, Cy5, and Cy7, Alexa Fluor 700, Atto 425, Atto 495, Atto 680, methylene blue, berberine, show a ratio of quantum yields in both solvents (ϕ_D/ϕ_H) from 1.1 to 2.6 but neither isotope exchange nor reduction of ESPT and/or ESPT is assumed to be present [14]. A possible explanation lies in energy transfer through dipole-dipole coupling between the excited molecules and water [28, 29]. Deuterium bonds are assumed to be slightly stronger than H bonds

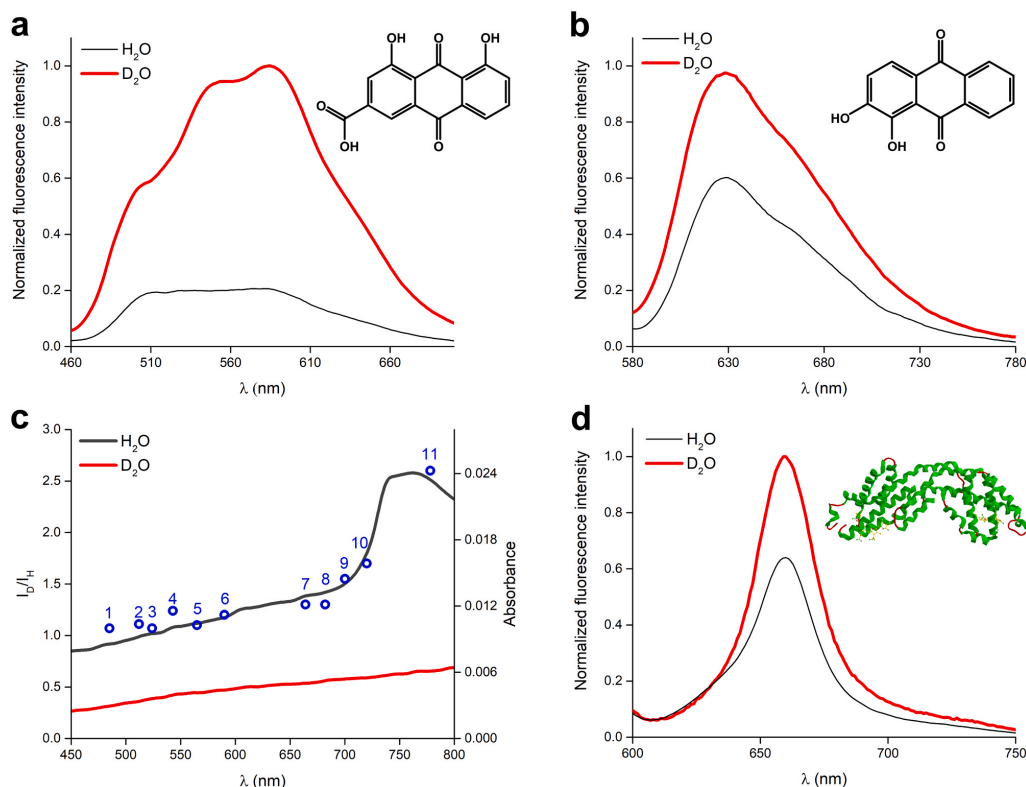


Fig. 3. (a) Emission spectra of anthraquinone rhein in H_2O (black line) and D_2O (red line) at pH (pD) = 9.5. Note the different shape of spectra. (b) Emission spectra of alizarin red S in H_2O (black line) and D_2O (red line) at pH (pD) = 9.5. Identical emission maxima and spectral shape indicate that eventual tautomerization is not influenced by the presence of D_2O . (c) The UV-Vis spectra of H_2O (black line) and D_2O (red line) together with the fluorescence intensity ratios (I_D/I_H) of chosen fluorophores (blue dots) in order of their emission wavelength (x-axis position). Fluorophores: 1 – Atto 425 (not buffered), 2 – fluorescein (pH(pD) = 12), 3 – Atto 495 (not buffered), 4 – berberine (pH(pD) = 7), 5 – Cy3 [14], 6 – Cy35 [14], 7 – Cy5 [14], 8 – methylene Blue (pH(pD) = 8.5), 9 – Atto 680 (pH(pD) = 7), 10 – Alexa Fluor 700 [14], 11 – Cy7 [14]. (d) Emission spectra and the structure of the fluorescent protein allophycocyanin (APC) in H_2O (black line) and D_2O (red line).

(due to the differences in zero-point energies) [30,31].

But at this point, it is necessary to take into account the strong absorption of H₂O in the visible light region (>500 nm) over D₂O (Fig. 3c), which is caused by intense vibrational transitions of light water as a higher mass of deuterium exhibit lower vibrational energies. A molecule of H₂O can effectively accept energy from an excited molecule if it is located in close vicinity of the fluorophore. Weak interactions such as hydrogen bonds (hydrogen bond assisted non-radiative deactivation) and other electrostatic interactions probably play a crucial role in the process of energy withdrawal [32]. Since absorption of H₂O increases with wavelength across several local maxima, the effectivity of the de-excitation process is more remarkable at longer wavelengths. The negligible absorption of deuterated water in the visible light region leads to significant differences in quantum yields and lifetime ratios, especially in cases of fluorophores emitting photons at longer wavelengths.

4. Applications

4.1. Dyes, labels, and probes

Adding (or substituting) D₂O in the solution enhanced the luminescence intensity of almost all studied dyes. The examples of commonly used extrinsic fluorescence markers are listed in Table 1. All our experiments have been measured in buffered solutions, as emission spectra and lifetimes are pH (and pD) dependent (Fig. 2a). Values of pD, measured by a glass electrode, were corrected according to literature [33,34]. To provide a comprehensive and thorough overview, we listed commonly used fluorophores in Table 1 that includes values obtained within this study along with the values published elsewhere.

Interestingly, the widely used and highly fluorescent DNA intercalating agents ethidium bromide and propidium iodide may increase their fluorescence intensity up to 200% in D₂O. However, even DNA-intercalated ethidium bromide exhibits almost a 100% increase of fluorescence signal (Table 1). A minor groove binder Hoechst 33258 also shows up to 60% better performance at neutral pH (pD) and Lucifer yellow, which is an up-and-coming cell label with numerous application in molecular biology, has an I_D/I_H ratio of 1.96 [35]. Based on the electron transfer mechanism, it was able to observe an increase in luminescence enhancement with the increasing emission maximum

wavelength, e.g., cyanine dyes Alexa Fluor 647 and Alexa Fluor 700 provided a 30% and 70% increase in I_D/I_H ratio, respectively. Similarly, the dyes Atto 425 and Atto 680 showed a 7% and 55% increase in fluorescence intensity in D₂O, respectively. Small enhancement can be probably assigned to a considerably high quantum yield of Atto 425 in normal water ($\phi_H = 0.9$, phosphate buffer pH = 7.4)[36]. Deuterated water increases also the luminescence intensity in well-known fluorophores such as fluorescein and rhodamine.

4.2. Biomolecules and important analytes

The rapid proton/deuteron exchange confirmed by NMR measurements substantially increased the quantum yield of tryptophan in deuterated solutions ($I_D/I_H = 2.3$) [9,10,39]. Hence, the fluorescence intensity of widely used fluorescent proteins could be influenced by the presence of D₂O (Fig. 3d) [40]. Although the primary mechanism responsible for luminescence enhancement is likely the rapid proton/deuteron exchange, it should be pointed out that a complex structure of proteins and their ability of interaction at various levels allow for contributions from other processes. (Fig. 4).

The fluorescence signal of selected pharmaceutical compounds from classes of anthraquinones, anthracyclines and similar might be enhanced by substituting light water for a heavy analogue, see Table 2. Accordingly, the highest fluorescence intensity increase was observed at compounds with a high number of hydroxyl and amino groups [41]. Certain enhancement of fluorescence intensity was also recorded for molecules containing thiol and amide groups (Tables 2, S2 and Figs. S11 and S12).

4.3. Capillary electrophoresis with fluorescence detection and separation techniques

Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) seems to be an ideal candidate for future applications regarding H₂O/D₂O substitution due to minimal volumes of chemicals necessary for analysis. Separation and detection of anthraquinone and anthracycline (doxorubicin and rhein) aimed to compare the limits of detection (LOD) in both solvents (50 mM sodium borate background electrolyte in H₂O or D₂O at pH (pD) = 9.5). Calculated LOD (the three-sigma method) for doxorubicin was found to be 9.0×10^{-9} M and 4.5×10^{-9} M in H₂O and D₂O, respectively. Rhein showed LOD to be 1.2×10^{-8} M in H₂O and 5.2×10^{-8} M in D₂O. The employment of deuterated water improved LOD 2.0-fold for doxorubicin and 4.3-fold for rhein. Higher intensities of fluorescence were accompanied by changes in migration times and higher viscosity, and lower conductivity of D₂O led to a lower electrophoretic current. A similar approach can be applied in reverse-phased chromatography; however, the solvent consumption is much higher and not necessarily cost-effective [15].

4.4. Flow cytometry

Flow cytometry is a unique technique mostly used to counting and sorting of cells and often is coupled with fluorescence detection. Cells are treated with fluorescent labels or probes before experiments, and the procedure is usually provided in a buffered aqueous solution. Usage of deuterated water for flow cytometry experiments was firstly described by Sailer et al. [42] The authors tested a series of DNA dyes on apoptotic subpopulations of HL-60 cells and found that non-intercalating probe mithramycin showed substantial enhancement of fluorescence signal (150%) in D₂O buffers. They demonstrated that also fluorescence signal of other dyes such as 7-aminoactinomycin D (34.5%), ethidium homodimer II (63.0%), TOTO (7.7%), and YOYO (9.1%) could be enhanced when are treated by D₂O-buffers. To verify the enhancement of fluorescence for other types of labels, we carried out flow cytometry experiments using human leukaemia cells HL-60 labelled by Calcein^{AM} and doxorubicin. The fluorescence signal of samples treated by heavy water

Table 1
Selected fluorescent dyes, probes and labels in D₂O and H₂O.

Compound	I_D/I_H	τ_D/τ_H	pH	Reference
fluorescein	1.70	1.64	pH (pD) = 2.0	This work
	1.11	1.07	pH (pD) = 12.0	
	3.81	–	Not buffered	[15]
rhodamine 6G	1.09	1.07	NaOH	[37]
rhodamine B	1.28	–	Not buffered	This work
Atto 425	1.07	1.04	Not buffered	This work
Atto 465	1.16	–	Not buffered	This work
Atto 665	2.09	2.05	Not buffered	[13]
Atto 680	1.55	–	pH (pD) = 7.0	This work
Alexa Fluor 488	1.15	–	pH (pD) = 7.0	This work
Alexa Fluor 647	1.30	1.20	pH = 7.5	[14]
Alexa Fluor 700	1.70	1.70	pH = 7.5	[14]
ethidium bromide	3.10	3.50	Not buffered	[12]
ethidium bromide + DNA	1.98	–	pH (pD) = 7.0	This work
propidium iodide	2.77	–	pH (pD) = 7.0	This work
calcein	3.10	–	pH (pD) = 7.0	This work
doxorubicin	2.87	4.04	Not buffered	This work
lucifer yellow	1.96	2.02	Not buffered	[38]
Hoechst 33258	1.60	–	pH (pD) = 7.0	This work
Cy3	1.10	1.00	pH = 8.5	[14]
Cy5	1.30	1.20	Not buffered	[14]
Cy7	2.60	2.40	Not buffered	[14]
nile blue	1.37	–	pH (pD) = 7.0	This work
coumarin 153	1.99	1.92	Not buffered	[16]
oxazine 170	1.94	2.28	Not buffered	This work
alizarin red S	2.56	–	pH(pD) = 3.0	This work

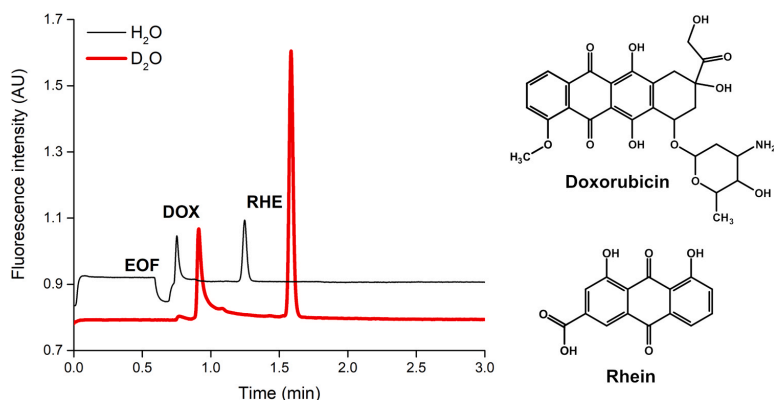


Fig. 4. CE-LIF Separation of doxorubicin (anthracycline) and rhein (anthraquinone) in H₂O (black line) and D₂O (red line) buffers.

Table 2

Fluorescence properties of selected analytes in D₂O and H₂O.

Compound	Class	I _D / I _H	pH	Reference
tryptophan	amino acid	2.30	pH = 7.0	[10]
tyrosine	amino acid	1.25	Not buffered	[7]
GFP	fluor. protein	1.54	pH = 7.4	[40]
mCherry	fluor. protein	1.33	pH(pD) = 7.0	This work
allophycocyanine	fluor. protein	1.55	pH(pD) = 7.0	This work
aesculetin	lactone	1.86	pH(pD) = 7.0	This work
riboflavin	vitamin	1.18	pH(pD) = 7.0	[15]
berberine	alkaloid	1.24	pH(pD) = 7.0	This work
rhein	anthraquinone, laxative	2.74	Not buffered	This work
tetracycline	tetracycline antibiotic	2.27	pH(pD) = 3.0	This work
albendazole	antiparasitic pharm.	1.64	pH(pD) = 3.0	This work
rifaxamin	aminoglycoside antibiotic	2.24	pH(pD) = 10.0	This work
paracetamol	analgesics and antipyretics	1.20	0.2 M NaOH (NaOD)	This work

increased in both cases (30% and 72% for Calcein^{AM} and doxorubicin, respectively). No alternative behaviour of the methodology was observed in the deuterated buffer experiment. As many fluorophores capable of luminescence enhancement in D₂O (Tables 1 and 2) are commonly used in flow-cytometry, routine employing of heavy water when the fluorescence signal requires enhancement is feasible.

4.5. Fluorescence microscopy

Fluorescence imaging of fixed (or live) stained samples is still the most broadly used technique across the field. The main advantage is in its simplicity and versatility as one does not have to use a specifically equipped microscope to visualise and quantify the fluorescence signal. The process of staining has its limitations and pitfalls, but the main goal is to have the brightest fluorescence signal as possible in order to distinguish the signal from the background, which could often originate from intrinsic fluorescence. Fig. 5(a, b) demonstrates the difference in the fluorescence signal intensity for mCherry staining in the case of cells treated with H₂O and D₂O buffer. The D₂O significantly increases the brightness of the fluorophore, resulting in a cleaner final image with better SNR and lower background. The difference in brightness corresponds to higher intensity (30%) recorded by steady-state measurements (Table 2) for mCherry samples in D₂O. In the case of using sensitive or less stable fluorophores, the deuterated buffer allows for

lower power of excitation light to avoid photobleaching. This is also essential in the case of live-cell imaging, where the lowest possible excitation power is necessary.

4.6. Super-resolution imaging

The performance of advanced fluorescence techniques such as super-resolution microscopy imaging is dependent on the number of photons emitted from each fluorophore. The addition of D₂O offers a cost-effective way to enhance the fluorescence quantum yield of commonly used dyes without changes in established methods.

All of the approaches and techniques used in super-resolution microscopy are highly dependent on the stability and intensity of each individual fluorophore which is the key to successfully employ single-molecule techniques. All of the methods are also highly-time demanding and require precise sample preparation. The goal is to visualise live-cell experiments at the highest possible resolution that is very challenging in case of preserving the cell's viability and stability of fluorophores. The addition of D₂O into super-resolution microscopy can be therefore highly beneficial and could even make some experiments possible to perform and analyse the data that would be otherwise buried in low SNR or fluorophore stability or damaging and affecting the cell's viability, etc.

Lee et al. used oxazines (a series of Atto dyes) and demonstrated that fluorescence quantum yield in D₂O could be doubled in comparison to H₂O [13]. Interestingly, Klehs et al. used a series of cyanine dyes for a dSTORM imaging experiment with an even 150% higher yield for Cy7 dye in D₂O buffer [14]. Deuterated water was also successfully combined with fluorescent proteins [40]. The steady-state experiments (Table 1) indicated that other super-resolution imaging dyes might be further enhanced by employment of D₂O [43].

4.7. Fluorescence correlation spectroscopy

Since we have not found any literature about an FCS experiment in the deuterated solvent, we tested four different dyes to verify the possible benefit. Alexa Fluor 488, Alexa Fluor 594, Propidium iodide, and APC protein were selected as typical dyes used in FCS experiments. Fig. 5c represents their counts per second per molecule (CPM) values for H₂O and D₂O buffers. The average increase in CPM of all analysed fluorophores is 50%. If we consider that CPM value is proportional to the signal to noise ratio, and the analyte concentrations are extremely low to achieve single-molecule precision, any higher CPM value will significantly improve the outcome of an FCS measurement.

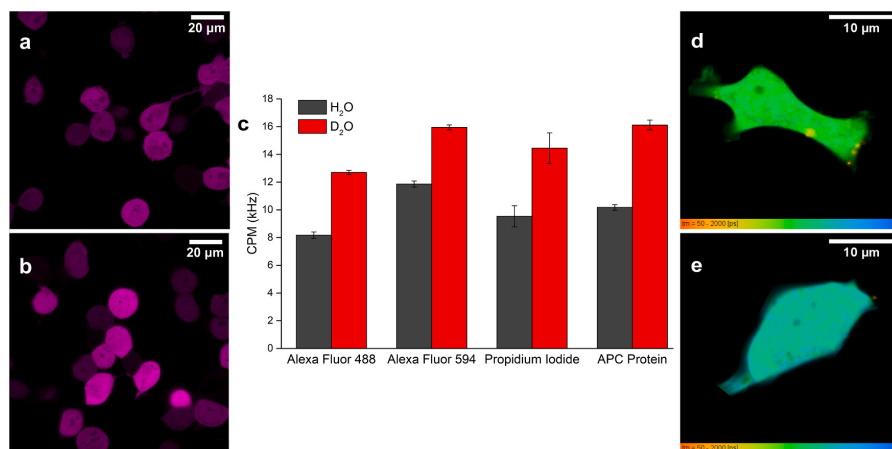


Fig. 5. (a, b) Live-cell wide-field fluorescence microscopy of HEK293T cells transiently expressing mCherry fluorescent protein. Cells were transfected one day prior to observation. During the imaging the cells were incubated in a microscopic chamber at 37 °C and 5% CO₂ to preserving the viability and were treated in PBS buffer prepared in H₂O (a) or D₂O (b). The D₂O buffer significantly increased the fluorescence intensity of mCherry fluorescent protein. (c) FCS measurement of four different fluorophore samples in H₂O (grey bars) and D₂O (red bars) buffers. CPM (counts per second per molecule) values demonstrate the quality of samples. Error bars represent the standard deviation of ten individual measurements. (d, e) FLIM images of HEK293T cells expressing a mCherry fluorescent protein. The average fluorescence lifetime is 970 ps for cells treated with H₂O (d) and 1327 ps for cells treated with D₂O (e). Colours in images represent a time scale of fluorescence lifetimes 50–2000 ps.

4.8. Fluorescence-lifetime imaging microscopy

FLIM analysis of a mCherry fluorescent protein was measured in transiently transfected HEK293T cells. Fig. 5(d, e) shows the fluorescence lifetime difference in cells treated and buffered in H₂O or D₂O. In the case of using a D₂O buffer, the fluorescence lifetime is prolonged by 37% on average. Fluorophores with higher quantum yield and longer fluorescence lifetime are generally preferable for lifetime measurements to provide a better imaging contrast or lower the demand on the laser pulse width.

5. Conclusions

We quantified and reviewed how the addition of D₂O affects the properties of fluorophores used in fluorescence-based methods. We determined the possible mechanisms responsible for the fluorescence intensity and lifetime increase caused by heavy water substitution. The extent of D₂O induced enhancement of fluorophore properties may be tuned accordingly to the practical demands of the discussed analytical techniques including quantification standards and detection limits. Our experimental work and available published data support the view that D₂O enhances the fluorescence signal due to the (i) exchange of labile hydrogen for heavier deuterium and (ii) the reduction of energy transfer from excited fluorophores to H₂O molecules.

In summary, we suggest that using deuterated water is an economically reasonable approach how to expand the applicability of fluorescence-based methods, as D₂O causes a higher signal-to-noise ratio, lower detection limits, decreased consumption of fluorophore, and reduced excitation laser power. Thus, using D₂O brings significant detection benefits especially in advanced experiments where signal enhancement might be essential for successful and precise examination.

CRedit authorship contribution statement

J. Kučera: experiment designing, fluorescence measurement, writing. **O. Peš:** MS measuring, writing. **T. Janovič** and **C. Hofr:** microscopic experiments designing and writing the microscopic section.

L. Kubinyiová: MS and fluorescence experiments. **J. Tóth:** fluorescence experiments. **Š. Kaňa:** fluorescence and CE-LIF experiments. **P. Táborský:** writing (original manuscript), experiment designing and supervising the study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.snb.2021.131029](https://doi.org/10.1016/j.snb.2021.131029).

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Review article 1

Quantitative Biology of Human Shelterin and Telomerase: Searching for the Weakest Point

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Review

Quantitative Biology of Human Shelterin and Telomerase: Searching for the Weakest Point

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Abstract: The repetitive telomeric DNA at chromosome ends is protected from unwanted repair by telomere-associated proteins, which form the shelterin complex in mammals. Recent works have provided new insights into the mechanisms of how human shelterin assembles and recruits telomerase to telomeres. Inhibition of telomerase activity and telomerase recruitment to chromosome ends is a promising target for anticancer therapy. Here, we summarize results of quantitative assessments and newly emerged structural information along with the status of the most promising approaches to telomerase inhibition in cancer cells. We focus on the mechanism of shelterin assembly and the mechanisms of how shelterin affects telomerase recruitment to telomeres, addressing the conceptual dilemma of how shelterin allows telomerase action and regulates other essential processes. We evaluate how the identified critical interactions of telomerase and shelterin might be elucidated in future research of new anticancer strategies.

Keywords: telomerase; shelterin; telomere; quantitative biology; protein-protein interaction; protein-DNA interaction; assembly; inhibitor; anticancer

1. Introduction

Telomeres are an excellent example of how a combination of random mutations and selection over hundreds of millions of years solves challenges that appear during evolution.

The first challenge is that the ends of linear DNA of eukaryotic chromosomes cannot be fully replicated. The incomplete replication of the 3' end of DNA results in an overhang of single-stranded telomeric DNA. The lagging strand, and thus the length of telomeric DNA, shortens every round of replication (50–200 per each DNA synthesis). Even though the gradual shortening of telomeric DNA could be used as a molecular mark of the number of divisions that a cell has undergone, the gradual loss of DNA sequence should be prevented in fast-dividing germ line cells and stem cells [1]. The end-replication problem is solved by the action of telomerase. Telomerase is a nucleoprotein complex, containing an RNA template, that synthesizes tandem repeats of telomeric DNA and compensates for the DNA erosion.

Another challenge is that chromosomes have to be protected against end-to-end fusions and undesirable recognition of telomeric DNA ends by double-strand DNA break repair pathways. To achieve chromosome protection, a protein complex called shelterin binds telomeric DNA. Shelterin not only protects the DNA ends, but also regulates the access of processing enzymes, such as telomerase and helicases, to telomeric DNA.

Finally, the fact that telomerase is also active in tumor cells, contributing to their immortalization, could be turned into an advantage in the treatment of cancer if we could deactivate or slow down telomerase, specifically in tumor cells, we would be able to eradicate tumor cells more effectively.

In this review, we focused on new findings on how shelterin assembles and mediates telomerase recruitment to telomeres and how the interaction map of shelterin and telomerase could be employed in anticancer strategies of new biomolecular therapies.

2. Shelterin Structure and Binding Features

Human chromosome ends are bound to, and protected by, shelterin complexes. Shelterin consists of six different proteins: telomeric repeat-binding factor 1 (TRF1; also known as TERF1), telomeric repeat-binding factor 2 (TRF2; also known as TERF2), repressor and activator protein 1 (RAP1; also known as TERF2IP), TRF1-interacting nuclear protein 2 (TIN2; also known as TINF2), protection of telomeres 1 (POT1), and TPP1 (derived from its former names TINT1, PTOPI, PIP1; also known as ACD).

Importantly, a stable shelterin complex is formed solely through protein-protein interactions of the shelterin subunits, and DNA is not required [2,3]. As shelterin functions on telomeric DNA that contains a single-stranded overhang, the shelterin anatomy can be divided into two parts that associate with either double- or single-stranded telomeric DNA (Figure 1a). The part of shelterin that binds the double-stranded telomeric DNA consists of TRF1, TRF2, RAP1, and TIN2 [4]. Both TRF1 and TRF2 bind telomeric DNA duplexes specifically through the Myb domain [5]. The next subunit, RAP1 interacts solely with TRF2. RAP1 binds TRF2 tightly with an affinity similar to the affinity of TRF2 to DNA [6]. RAP1 also prevents the positively charged basic domain of TRF2 from sequence-nonspecific DNA binding, thereby increasing the specificity of TRF2 for human telomeric TTAGGG repeats [6]. TIN2 interconnects TRF1 and TRF2, and enhances TRF2 stability at telomeres [7,8].

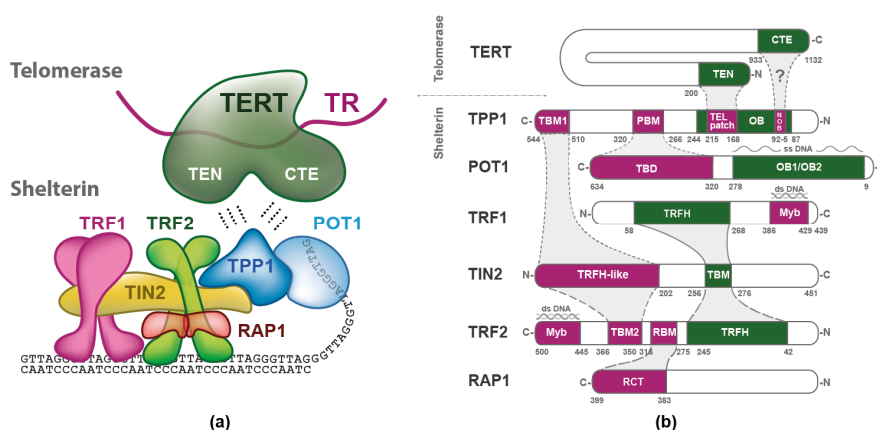


Figure 1. Arrangement and interaction domain map of shelterin and telomerase: (a) Schematic description of telomerase and shelterin subunits. TERT is the catalytic subunit with telomerase essential N-terminal (TEN) domain and C-terminal extension (CTE) domain, TR is the RNA subunit of telomerase. Shelterin subunits TRF1, TRF2, and RAP1 are shown as dimers, and the other shelterin subunits are illustrated as monomers. (b) The interaction map of the telomerase TERT subunit and the six human shelterin subunits are depicted. The Myb/SANT domains of TRF1 and TRF2 recognizing double-stranded DNA are indicated with Myb. The OB1/OB2 domain of POT1 recognizing single-stranded DNA. TBD is the TPP1 binding domain of POT1. PBM is POT1 binding motif of TPP1. The OB domain of TPP1 contains two regions: the TEL patch that binds TEN domain of telomerase and the NOB region that probably binds CTE domain of telomerase, denoted by “?” [9]. TBM of TIN2 binds the TRFH domain of TRF1 and TRF2. TBM1—TIN2-binding motif of TPP1, TRFH—dimerization domain of TRF1 and TRF2, TRFH-like—TIN2 domain that binds TPP1 and TRF2; TBM2 binds TRFH-like binding motif of TIN2, and TBM2—TIN2-binding motif of TRF2. RBM—RAP1 binding motif of TRF2. RCT is the RAP1 C-terminal domain that binds RBM of TRF2. The more solid the outline between the interacting domains, the higher their mutual affinity [7,10].

The part of shelterin that is associated with single-stranded DNA consists of the heterodimer POT1 and TPP1. POT1 binds single-stranded telomeric DNA via two oligonucleotide/oligosaccharide binding (OB) folds (Figure 1b) [11,12]. POT1 recognizes the sequence 5'-TTAGGGTTAG and binds it with a nanomolar affinity when located at the 3' end, or with a slightly lower affinity when the recognition sequence is at an internal position [11,13].

Recently, the Cech laboratory carried out biophysical studies describing how the single-stranded and double-stranded DNA binding parts of shelterin contribute to the DNA-binding affinity of shelterin [14]. Lim et al. designed a DNA substrate, comprising both single-stranded and double-stranded telomeric regions separated by 8 bp of random double-stranded DNA. When the double-stranded telomeric sequence on DNA was replaced with a random DNA sequence, the original nanomolar binding affinity of shelterin core proteins TRF2-TIN2-TPP1-POT1 dropped 6-fold. Similarly, when the part of the single-stranded DNA with telomeric sequence was replaced with a random DNA sequence, the binding affinity of the core shelterin dropped 35-fold. Interestingly, after removal of either TRF2 or POT1 from the core shelterin, its binding affinity to the DNA substrate decreased 50-fold and 14-fold, respectively [14]. These results suggest that the removal of TRF2 or POT1 from shelterin decreased its DNA binding affinity. The quantitative experiments also showed the contribution of TRF2 and POT1 to the specific recognition of telomeric DNA by shelterin. In addition, this is in accordance with the general view that DNA-binding proteins associate with DNA nonspecifically at first and seek their recognition sequence and optimal binding site through specific interactions subsequently [15].

Shelterin complexes appear to bind telomeric DNA without recruiting each other *in vitro* [2], which is in accordance with the observation that shelterin finds its binding sites through a diffusive 3D search, as well as by a 2D search along the DNA [2,4]. Telomeric single-stranded overhang can fold back and form a telomeric loop (t-loop). This structure was first observed by electron microscopy [16] and later confirmed by super-resolution microscopy [17]. T-loops can physically hide the very ends of eukaryotic chromosomes from DNA repair pathways and telomerase [18]. TRF2 is essential for the formation of the t-loop [17]. In addition, POT1 stabilizes t-loop formation [19]. T-loops are protected and maintained by shelterin complexes [20]. The putative arrangement of shelterin on t-loops is shown in Figure 2. Recently, F. Erdel et al. have investigated the localization of shelterin on telomeric DNA *in vitro*. They discovered that shelterin rapidly and primarily recognizes telomeric repeats by a three-dimensional diffusive search. The complex remained bound to telomeric DNA more than 30-fold longer in comparison with non-telomeric DNA—it persisted bound for ten minutes without dissociating from telomeric DNA. According to their electron microscopy experiments, there is approximately one shelterin complex per 100 bp of telomeric repeats [2].

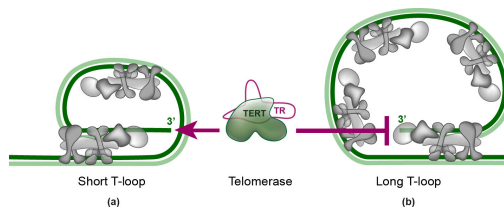


Figure 2. T-loop formation as a factor for telomere length regulation. The 3' overhang invades the double-stranded telomeric DNA and, by base-pairing with the C-strand, displaces the G-strand, which causes the end of the telomere to be tucked in. Double-stranded DNA-binding proteins of shelterin, TRF1 and TRF2, mediate and fold DNA to the t-loop. The amount of shelterin that is bound to telomeres is dependent on the t-loop length. (a) A short t-loop with less abundant shelterin has a lower probability that shelterin covers and hides the 3' overhang of telomeric DNA. Telomerase can extend telomeric DNA. (b) The long t-loop is occupied by more shelterin complexes, so the probability that the 3' overhang is covered and hidden is higher. Telomerase access to the 3' end single-stranded DNA is blocked and no DNA extension occurs.

3. Telomerase

Telomerase consists of two main subunits. One of the subunits is the telomerase reverse transcriptase (TERT). TERT is recruited to telomeres during the S-phase of the cell cycle. The interaction of telomerase with shelterin is mediated by TPP1 (Figure 1). The other subunit of telomerase is telomerase RNA (TR)—a 451 nucleotides-long oligomer—which binds the TERT subunit by the template-pseudoknot domain and CR4-CR5 domain [21–24]. TR has also been shown to contribute to TERT processivity, as TR also provides a template boundary element that limits the extent of reverse transcription [25,26]. The t-loop model of telomere and shelterin arrangements suggests that telomerase access to single-stranded telomeric 3' overhang contributes to the regulation of the telomere length maintenance (Figure 2) [20].

Telomerase is closely associated with cancer and other diseases. Telomerase is extremely important for tumorigenesis—telomerase is activated in 90% of all malignant tumors [27]. In many cancer cells, mutations in the promoter of the gene that encodes for telomerase reverse transcriptase (TERT) causes higher expression rate of TERT [28,29]. In addition, mutations with loss-of-function in either TERT or TR have been associated with dyskeratosis congenita, aplastic anemia and pulmonary fibrosis [30].

3.1. Structure of TERT and TR

The TERT catalytic core (1132 amino acids) consists of four major domains: the telomerase essential N-terminal (TEN) domain, the high-affinity RNA-binding domain (TRBD), the reverse transcriptase (RT) domain, and a C-terminal extension (CTE), analogous to a polymerase thumb [31–33]. The 3' ends of TR contain an H/ACA Cajal box (CAB) motif that can bind various telomerase accessory proteins. TR is associated with RNA-binding proteins dyskerin, NHP2, NOP10, and GAR1, which ensure TR stability, 3D structure of RNA, and telomerase ribonucleoprotein biogenesis [34,35].

Recently, Nguyen et al. showed in detail the cryo-EM structure of the active TERT protein subunits and TR, illuminating the structural basis of TR motif functions and human disease mutations that cause telomerase deficiency. They used transient transfection of HEK 293T cells with vectors expressing TERT and TR to prepare enough telomerase complexes for cryo-EM. To determine the telomerase structure at subnanometer resolution, they fit EM densitograms with available high-resolution structures and homology models of telomerase complex components [36]. The structure data supports the hypothesis that telomerase from various organisms functions in a monomeric state [37,38]. The monomeric state of telomerase, consisting of one TERT and one TR, is in agreement with suggestions of an evolutionarily-conserved single-TERT catalytic core [36,37,39,40].

The TEN domain of TERT has been implicated in providing the 'anchor site' that binds telomeric DNA upstream from the primer-template interaction [41–43]. The TEN domain also contains the DAT motif, which has been postulated to be involved in telomerase recruitment to the shelterin complex [44]. In particular, the mutation K78E in the DAT domain was shown to prevent telomerase recruitment by TPP1 [45].

There are two TR domains critical for telomerase activity: the first is the domain with the template and adjacent pseudoknot (t/PK), and the second is the conserved regions 4 and 5 (CR4/5), consisting of a branched junction of stems P5 and P6 with the activity-critical stem-loop P6.1 [46,47]. The pseudoknot and triple helix structure contribute to catalysis, perhaps by orienting the primer-template duplex in the enzyme active site [48–50].

3.2. The Function of TERT and TR

In vivo, the telomerase action on DNA can be described by: (i) binding frequency—the number of bindings to telomeric DNA per cell cycle and (ii) repeat addition processivity—the number of newly synthesized telomeric DNA repeats per association event [45].

Telomerase interacts with the chromosome end in two different ways, namely: (i) short “probing” interactions—dynamic interactions with telomeres and (ii) “static” interactions—sufficiently long interactions that allow telomere elongation. TERT requires TPP1 binding for both interaction modes [45,51,52]. Recently,

experiments with live cell imaging demonstrated that long-static interactions of telomerase occur only when telomerase pairs with a single-stranded DNA overhang [45].

In vivo, telomerase can add around 50–60 nucleotides to most chromosome ends in a single processive step [53]. In vitro, telomerase has a higher processivity and can recover 100 nucleotides lost from replicated chromosome ends in a single elongation event [33]. If TERT is substantially overexpressed, the TR subunit becomes limiting, so telomerase activity increases only ~1.5–2-fold [45,52,54,55].

The association of TCAB1 with TR is required for telomerase localization in Cajal bodies [56]. Cajal bodies are regions within the nucleus that are rich in proteins and RNAs that are involved in the assembly of ribonucleoproteins. Schmidt et al. revealed how telomerase explores the nuclear space to search for telomeres through dynamic probing interactions using live cell imaging. More than 85% of unbound telomerase moved fast (diffusion coefficient $D > 0.3 \mu\text{m}^2/\text{s}$) [51]. After association with telomeres, TERT slowed down and appeared in the highly static state with a diffusion coefficient more than 100-fold lower. Similarly, when TERT, which contains TR, is near Cajal bodies, TERT appeared in the static state again [51]. Thus, recent live cell imaging studies suggest that telomerase recruitment to telomeres is driven by dynamic interactions between the rapidly diffusing telomerase and the chromosome ends [51].

4. Telomerase Recruitment via Shelterin

Telomerase recruitment to telomeres involves the deployment of telomerase from Cajal bodies to telomeres, which is driven by the interaction of telomerase with TPP1 [57].

The initial in vivo studies suggested that telomerase interacts through TEN and the CTE domain with the OB domain of TPP1 [58]. Specific loop residues within the OB-fold that are critical for the association of TPP1 and telomerase have been identified by structural studies and confirmed by mutation analysis of the OB domain of TPP1 with subsequent quantitative analysis of telomerase activity [59]. The experiment identified seven residues of the OB domain of TPP1 that are critical for telomerase recruitment. The critical residues of TPP1 OB domain define the TEL (TPP1 glutamate (E) and leucine (L)-rich) patch. The four glutamic acid residues give the TEL patch a negative charge. Recently, the Nandakumar laboratory suggested that the highly basic TEN domain of telomerase binds into the acidic pocket of the TEL patch, while the hydrophobic pocket of the N-terminus of the OB domain is occupied by residues residing elsewhere in TERT, probably in CTE [9]. Telomerase recruitment also depends on TIN2, as TIN2 is essential for TPP1 recruitment to telomeres [60].

Recently, the Cech laboratory demonstrated that the full-length POT1, together with the full-length TPP1 protein and the TIN2 subunit, increases telomerase processivity about 3-fold. Lim et al., in their recent study, proposed that the recruitment of telomerase to telomeric DNA could occur not only through the TPP1-POT1 heterodimer, but also through the TPP1-TIN2-TRF1 or the TPP1-TIN2-TRF2 complexes that are associated with the double-stranded DNA-binding part of shelterin [14].

Shelterin proteins TRF1 and TRF2 might play an important role in the protein-counting model of telomere length regulation via telomerase. This model involves two different states: (i) “open” state—telomerase can elongate the telomeres and (ii) “closed” state—telomerase access is blocked (longer telomeres can recruit more TRF1 and TRF2) [61]. Switching between these two states is driven by TRF1 and TRF2 that act as negative regulators of telomere length [61]. Originally, the negative feedback loop for telomere length regulation was suggested in yeast by the Shore laboratory [62]. The protein-counting model is also applicable for t-loop formation and telomere protection by bound shelterin complexes (Figure 2).

5. Quantitative Description of Interactions within Shelterin

Shelterin, as a multi-subunit protein complex, is formed through dynamic interactions between its protein subunits. It is known that both TRF1 and TRF2 proteins interact with TIN2, which is the key bridging factor that brings together shelterin subunits attached to double-stranded DNA and subunits attached to the single-stranded DNA overhang.

TPP1 interacts with TIN2, and this particular interaction is suggested to be the essential step in high-order shelterin assembly. The TRF2-TIN2-TPP1 interaction was thoroughly studied by Hu et al. in 2017, where they revealed the importance of TIN2 interactions with TPP1 together with TRF2. They found that the TBM1 (TIN2-binding motif) of TPP1 and TRF2 (TBM2) bind to different sites in TIN2 (2–202aa), where a long alpha helix of TIN2 interconnects TPP1 and TRF2. Thus, it was proposed that the alpha helix acts like a seesaw where TPP1 is on one end and TRF2 is on the other. This concurrent interaction allosterically regulates optimal TIN2 ability to bind TPP1 and TRF2 simultaneously [10]. Recently, we found that, at the single-molecule level, TRF1 is able to replace TRF2 from TIN2 when TPP1 is absent. Thus, TPP1 is required for stable TRF1-TIN2-TRF2 complex formation [3].

Structural studies of shelterin subunits have to be accompanied by affinity studies to reveal structure-function relationships among shelterin subunits. In addition, the description of mutual affinities of shelterin subunits can determine limiting steps in shelterin assembly. Most of the available data are results of binary interaction studies of shelterin components, often limited to their interaction domains only [20,63,64]. When Lim et al. thoroughly investigated the reconstitution of human core shelterin complexes, they revealed a 2:1 binding stoichiometry for TRF2 and TIN2, respectively [14].

Rice et al. described the interaction of TPP1 (255–337aa) and POT1 (330–634aa) by isothermal titration calorimetry and revealed that $K_d = 120$ nM [65]. Previously, it has been shown by the Cech laboratory that TPP1 promotes POT1 binding to single-stranded telomeric DNA [66–68]. Accordingly, the Skordalakes laboratory used fluorescence polarization to demonstrate that full-length POT1 binds DNA with a K_d of approximately 20 nM, but, when POT1 is complexed with TPP1 (87–544aa), the affinity increases more than 3-fold [65].

TIN2 has two binding sites with different binding preferences for TRF2. According to Hu et al., 2017, the domain interaction studies using fluorescence polarization revealed that $K_d = 2.7$ μ M for TRF2's TBM2 domain binding to TIN2 (2–202aa)—truncated protein variants, which covers the TIN2 interaction region for binding TRF1 and TRF2. When TIN2 (2–202aa) is complexed with TPP1's TBM1 domain before the interaction with TRF2's TBM2 domain, the affinity is increased 2.5-fold ($K_d = 1.1$ μ M). If we look at the binding of interacting domains the other way around, TIN2 (2–202aa) binds TPP1's TBM1 domain with $K_d = 0.52$ μ M, and $K_d = 0.14$ μ M when TPP1's TBM1 domain is complexed with TRF2's TBM2 domain [10]. TPP1 changes the structure of TIN2 and expands its binding capacity [3,10].

We have been studying mutual interactions of human telomeric proteins and DNA in our laboratory systematically [3,6,68,69]. Figure 3 summarizes the results of our binary interaction studies with proteins of human shelterin. Our recent thermophoresis measurements with full-length proteins have revealed that TRF1 binds to TIN2 with $K_d = 240$ nM, whereas TRF2 binds to TIN2 with $K_d = 360$ nM. (Figure 3). These data suggest that TRF1 shows greater binding affinity to TIN2 than TRF2. Based on our quantification of mutual interactions within shelterin, we propose that the limiting step for the complete shelterin complex assembly is the interaction of TIN2 and TPP1. The binding of full-length TIN2 and TPP1 (89–544aa) is characterized with the highest K_d value (2.8 μ M), i.e., the lowest affinity, measured by fluorescence anisotropy and thermophoresis. Thus, the interaction of TIN2 and TPP1 is the weakest interconnection within the shelterin complex. The interaction of TIN2 and TPP1 plays a crucial role in bridging shelterin parts attached to single and double-stranded telomeric DNA (Figure 1a, Figure 2, and Figure 3). It is important to point out that mutual affinity of two shelterin proteins might be potentiated by binding of other shelterin components.

The quantitative description of shelterin subunit interactions suggests that the weakest point is protein TIN2 and its TIN2-TPP1 interaction. In addition, the other weak point is the binding of telomerase by TPP1, as telomerase recruitment is a crucial step in the telomere length maintenance mechanism. Thus, TPP1 participates in the interactions that are limiting for shelterin assembly, on one hand, and telomerase recruitment, on the other hand.

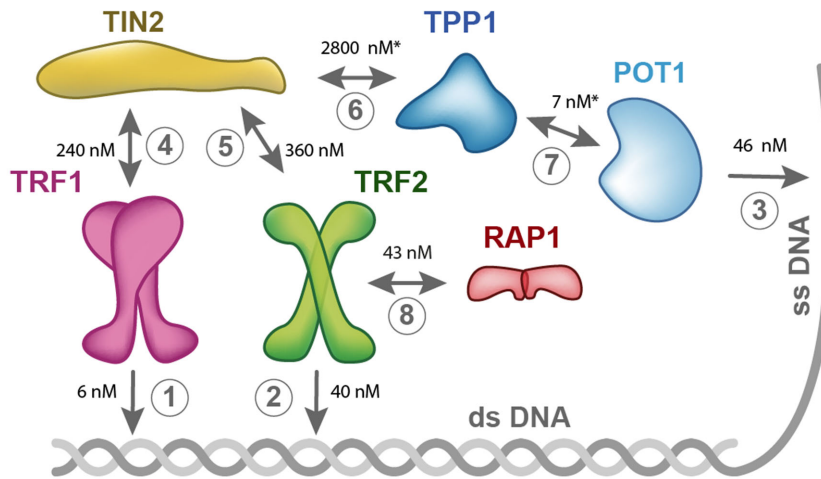


Figure 3. Binary interactions within shelterin measured in our laboratory. Binding studies with six shelterin proteins were carried out using fluorescence anisotropy (1, 2, 3, 7, 8) and thermophoresis (4, 5, 6). The asterisks denote that the K_d values were determined for truncated variant of TPP1 (89–544aa) and POT1 (312–634aa). All other quantifications of interactions were carried out with full-length proteins. The binding buffer was 50 mM sodium phosphate, pH 7.0, 50 mM NaCl. The binding temperature was 25 °C, except for 1 where 10 °C was used to secure TRF1 protein stability during measurements. K_d values were determined as the mean of at least three independent experiments with a relative standard deviation of less than 10%.

6. Implication for Anticancer Drug Design

The inhibition of telomerase activity may increase the sensitivity of tumor cells to chemotherapy, radiation, and other targeted therapies. In order to slow down telomere elongation, we could block the interaction between telomerase and shelterin. To impair the telomerase function completely, telomerase binding to DNA would have to be abolished. Two promising approaches are being studied: (i) block the TR-binding region of telomerase (e.g., with inhibitor GRN163L) or (ii) overprotect single-stranded telomeric DNA overhang by its hybridization with artificial T-oligonucleotides. Both inhibitory strategies have demonstrated promising anticancer activity in multiple cancer types via the induction of potent DNA damage responses [70,71].

However, the main cause of cancer cells' eradication is the shortening of telomeres, and not telomerase inhibition exclusively, as cell may initiate telomerase-independent ways of telomere length maintenance. When telomere length reaches a critically short length, cells enter replicative senescence [72]. The deleterious effect of telomere shortening in cancer cells can be triggered by treating cells with cytostatic drugs.

6.1. GRN163L (*Imetelstat*)

GRN163L (JNJ-63935937, also known as *Imetelstat*) has been abundantly studied as a competitive inhibitor of telomeric DNA binding [45].

GRN163L has a unique telomerase inhibition ability: it binds directly and tightly to telomerase TR template. The telomerase RNA subunit represents an attractive target for oligonucleotide-based inhibition. The RNA sequence of TR contains an 11-nucleotide-long template region for binding and extending telomeric DNA substrates that is easily accessible for hybridization with complementary oligonucleotides [73].

GRN163L contains a complementary sequence to human TR (5′-palmitate-TAGGGTTAGACAA-NH₂-3′). Thus, GRN163L serves as a direct antagonist to the telomerase RNA template TR [71,74,75].

GRN163L has high solubility in aqueous solutions, it is resistant to nucleases, shows high stability in acidic solutions or in the presence of metabolites, and demonstrates high thermal stability of duplexes formed with the complementary RNA strands [76–80]. Importantly, GRN163L has been shown to significantly suppress not only the growth of primary tumors, but also the spread and proliferation of metastases [71]. GRN163L progressively diminishes the amount of telomeric primer bound to telomerase, consistent with the hypothesis that GRN163L competes with single-stranded telomeric DNA for telomerase binding [45]. The inhibition of telomerase activity in cancer cells by GRN163L results in progressive shortening of telomeres and eventual senescence or apoptosis [80].

6.2. Clinical Studies of GRN163L

To date, GRN163L is in the second phase of clinical trials, according to the Drugbank database. Systematic studies of GRN163L may reveal other therapeutic effects beyond the inhibition of metastasis and cancer proliferation, for example, involving the immune system and reduction of angiogenesis. Reversible effects were revealed when stem cells were shortly treated with GRN163L in vitro [81]. Despite numerous experimental studies showing promising anticancer effects of GRN163L, FDA approval has not been received yet. It is probably because clinically relevant doses of GRN163L as a stand-alone therapy showed an elevated toxicity level [82,83]. Some patients with a pediatric brain tumor suffered from hematological toxicity of GRN163L—the most common side effects were thrombocytopenia, lymphopenia, and neutropenia [82]. GRN163L may have several side effects on the stem cell population [81]. On the other hand, GRN163L showed promising results in combination with radiation therapy, which may overall improve the therapeutic index [84–86]. Breast cancer cells treated with GRN163L showed enhanced sensitivity to ionizing radiation that reduced their survival by an additional 30% [84].

6.3. T-oligo Cancer Therapy

T-oligo (an oligonucleotide homologous to the 3′-telomere overhang) targets abnormal regulatory signaling pathways, including DNA damage responses (DDRs) in cancer cells [87–91]. It has no or minimal toxicity in mice, melanocytes, or other normal cells [74,91–94]. After exogenous addition and accumulation in the nucleus, T-oligos induce DDRs, such as apoptosis and cell cycle arrest, which can be facilitated by the ataxia telangiectasia mutated pathway and its effectors cdk2, E2F1, p53, p95/NBS1, and pRb in cancer cells [74,91,93–95]. T-oligo has anticancer activity in multiple cancer cell lines in vitro, such as breast, colorectal, lung, lymphoma, melanoma, ovarian, pancreatic, and prostate cancers [86–91,95,96].

Ivancich et al. suggested two main models of the mechanism of anticancer effects by T-oligos [97]. The first model—“exposed telomere mimicry model”—hypothesizes that DNA damage-sensing proteins detect the T-oligo that accumulates in the nucleus of a cancer cell due to its homology to the telomere. The second model—“shelterin dissociation model”—is where T-oligo interacts with shelterin proteins, which are dissociated from the telomere and thus T-oligo exposes the telomere overhang that can initiate DNA damage responses [97]. Despite the initial promising results, T-oligos have not proceeded to clinical trials yet [71].

7. Perspectives

So far, the quantitative descriptions of binding affinities among shelterin subunits suggest that the weakest point of shelterin assembly is the TIN2-TPP1 interaction. The other potential weak point is the TPP-mediated recruitment of telomerase. Hence, TPP1 is engaged in two events that are crucial for successful telomere length maintenance. Thus, TPP1 is a very promising target for future studies focused on regulation of telomere length homeostasis at the molecular level. So far, shelterin and telomerase were thoroughly studied mainly on naked DNA in vitro. Quantitative biology of telomeres

and associated nucleocomplexes could reveal new findings if we include nucleosomes into next investigations. Future quantitative studies of telomerase and shelterin associations and recruitment will contribute to further understanding of molecular origins of disease associated mechanisms and might be applied in the development of new molecular therapies. The preparation of a stable TEN domain of human telomerase in vitro seems to be a very challenging task that slows down further investigation leading to rational design of the inhibitor of telomerase recruitment via shelterin. The direct inhibition of telomerase catalytic activity is possible by GRN163L (Imetelstat) that is promising regarding the future development of combined biochemical approaches to eradicate tumor cells with less side effects.

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Review article 2

Transcriptional regulators of human oncoviruses – structural and functional implications for anticancer therapy

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SURVEY AND SUMMARY

Transcriptional regulators of human oncoviruses: structural and functional implications for anticancer therapy

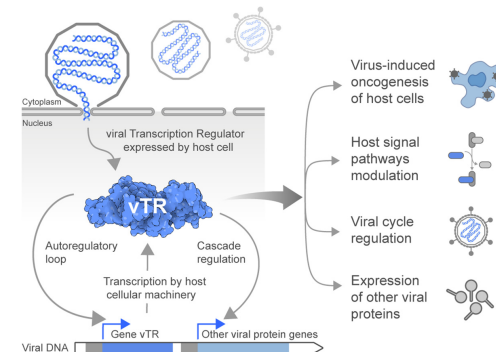
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ABSTRACT

Transcription is often the first biosynthetic event of viral infection. Viruses produce preferentially viral transcriptional regulators (vTRs) essential for expressing viral genes and regulating essential host cell proteins to enable viral genome replication. As vTRs are unique viral proteins that promote the transcription of viral nucleic acid, vTRs interact with host proteins to suppress detection and immune reactions to viral infection. Thus, vTRs are promising therapeutic targets that are sequentially and structurally distinct from host cell proteins. Here, we review vTRs of three human oncoviruses: HBx of hepatitis B virus, HBZ of human T-lymphotropic virus type 1, and Rta of Epstein–Barr virus. We present three cunningly exciting and dangerous transcription strategies that make viral infections so efficient. We use available structural and functional knowledge to critically examine the potential of vTRs as new antiviral-anticancer therapy targets. For each oncovirus, we describe (i) the strategy of viral genome transcription; (ii) vTRs' structure and binding partners essential for transcription regulation; and (iii) advantages and challenges of vTR targeting in antiviral therapies. We discuss the implications of vTR regulation for oncogenesis and perspectives on developing novel antiviral and anticancer strategies.

GRAPHICAL ABSTRACT



INTRODUCTION

Virus-associated diseases are the most challenging threats to humankind. In the last few decades, effective strategies to combat viral diseases have been developed and implemented worldwide. We have been able to eliminate several viral pathogens by vaccinations. An example of an effective elimination strategy is the successful eradication of smallpox by the World Health Organization (1), with the significant contribution of scientists and medical experts from the whole world (2,3). Unfortunately, there are viral diseases against which suitable vaccine approaches are insufficient, and we have to treat patients with ongoing infection. Several viruses are associated with cancer in humans. In 2008, there

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were an estimated 12.7 million new cases of cancers that accounted for 7.6 million deaths globally (4–6). One-sixth of cancer cases worldwide is linked to infectious agents, including viruses, bacteria and parasites (7). Oncoviruses cause 12% of all cancers (8) that account for >1.5 million new cases estimated per year.

Human oncoviruses comprise seven viruses: hepatitis B and C viruses (HBV and HCV), human papillomavirus (HPV), Epstein–Barr virus (EBV), human T-cell lymphotropic virus 1 (HTLV-1), Merkel cell polyomavirus (MCPyV) and human herpesvirus-8, also called Kaposi's sarcoma-associated herpesvirus (KSHV) (6). On one hand, human oncoviruses HPV, EBV, HTLV-1, MCPyV and KSHV contribute to oncogenesis directly via the translation of viral nucleic acid followed by viral protein expression. On the other hand, oncoviruses HBV and HCV contribute to the oncogenesis of host cells indirectly via chronic inflammation (9). Despite being DNA- and RNA-based viruses, oncoviruses share several common features: (i) Oncoviral infection is frequent, but the incidence of cancer development is rare – below 10 cases per 100 000. (ii) Oncoviruses that prevent early host cell lysis rather persist latently for a long time – up to several decades. Latent evasion strategy allows viruses to hide from the host immune response. Only a few specific cases, such as KSHV-induced Kaposi's sarcoma and HPV-induced cervical cancer, can arise as tumors within months of infection. (iii) Oncoviruses cannot trigger tumorigenesis without the incidence of additional risk factors, such as immune suppression, chronic inflammation, co-infection with other pathogens and host mutations (10). Thus, virus-induced infection is just one component of the complex carcinogenesis process.

So far, there are no effective cures specifically targeting viral-induced malignancies in humans (10–12). The most common antiviral drugs are inhibitors of viral DNA polymerases: nucleoside analogs such as acyclovir and acyclic nucleoside phosphonates such as cidofovir, adefovir and tenofovir (13). Clinically used antiviral drugs are accompanied by nephrotoxicity and reversible neurological toxicity, as in the case of acyclovir (14). If nucleoside analogs are used in chronic patients, a high incidence of acquired resistance is observed (15).

The design of new antiviral drugs might be focused on viral proteins. Targeting viral proteins yields specific, less toxic compounds with a narrow spectrum of antiviral activity compared to targeting host cell proteins (16). Therapeutically targeted viral proteins must be essential for the survival of viruses and multiplication of viral nucleic acids. Viral transcription regulators (vTRs) fully meet the exclusivity requirement for specific antiviral therapy, as vTRs are critical proteins in viral genome transcription. The inhibition of vTRs interrupts viral DNA or RNA synthesis that is indispensable for the complete viral life cycle. Despite the apparent importance of vTRs in human health and their potential as therapeutic targets, little is known about vTRs' structure, binding specificities to nucleic acids, host target proteins, transcriptional cofactors and precise molecular mechanisms of their action.

Roles of vTRs in the transcription of viral genomes: structural and functional properties of vTRs and research methodologies

In close cooperation with host transcriptional regulators, vTRs coordinate viral and human gene expression at several levels comprising transcription initiation, RNA polymerase recruitment, transcription elongation and chromatin organization. Accordingly, vTRs bind to nucleic acids either directly (Figure 1A) or indirectly through cofactors to modulate target gene expression. Additionally, vTRs modulate gene expression by targeting the transcriptional machinery or by altering chromatin states. The transcription machinery is eminently complex as viral genome transcription involves host cell pathways. Viral genes are usually transcribed in a particular temporal sequence. Tightly regulated transcription employs host cellular control mechanisms, such as signal transduction cascades that transmit specific environmental stimuli to the transcriptional machinery or cellular proteins that repress transcription. However, vTRs are critical components in establishing the order of viral gene transcription.

In general, to describe structure–function relationships of vTRs, a combination of biological (*in vivo*) and experimental (*in vitro*) approaches is required to find new structural motifs of vTRs that are essential for unique functions. To discover vTRs' gene targets and determine their activation or repression activities, *in vivo* functional studies employing reporter and overexpression assays are carried out. The vTRs that affect transcription indirectly have been identified and characterized through protein–protein interactions with host transcription factors and cofactors using immunoprecipitations followed by mass spectrometry (17). Next, chromatin immunoprecipitation (ChIP) in virus-infected cells or cells transfected with a particular vTR is employed to detect vTRs that are associated with DNA. Previous binding studies between vTR and host genome used ChIP followed by high-throughput sequencing (ChIP-seq) (18).

After identification of vTRs' binding targets, *in vitro* binding assays are performed for DNA–vTR and host protein–vTR complexes (19). To fully characterize DNA-binding and protein-binding specificities, a combination of methods such as electromobility shift assay (EMSA), fluorescence spectroscopy and isothermal titration calorimetry is employed (20,21). Analogically, *in vitro* binding assays establish the ability of vTRs to bind canonical DNA sequences and specific domains of host and viral proteins.

Recently, Liu *et al.* reviewed target genes, proteins and pathways regulated by particular vTRs and the relation of vTRs to human disease pathogenesis along with methodologies used for vTRs' characterization. Out of the total of 419 vTRs, 53 have been connected to cancer development. So far, 22 oncogenic vTRs have been confirmed to interact directly with nucleic acids (18). Here, we focus on three vTRs that interact with different primary targets contributing to oncogenesis: (i) HBx (HBV X protein) binding double-stranded DNA (dsDNA) and long non-coding RNA (lncRNA) that is involved in HBV – causing cirrhosis and hepatocellular carcinoma; (ii) HBZ (HTLV-1 bZIP factor) regulating transcriptional machinery indi-

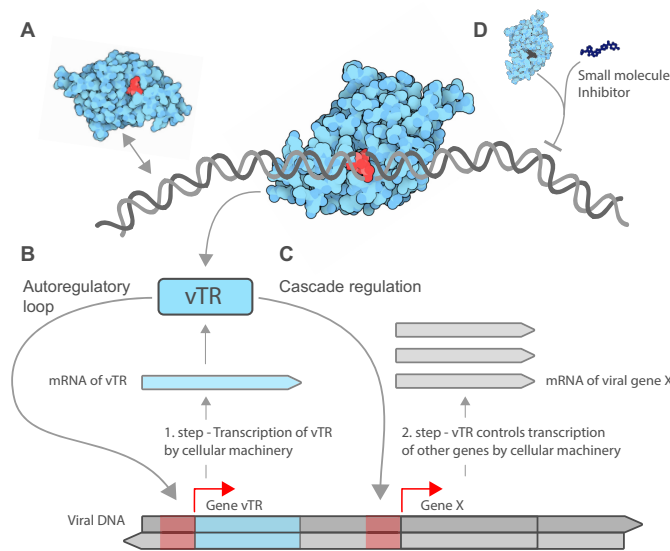


Figure 1. How vTRs stimulate transcription of viral genes. (A) vTRs act in concert with the host cellular transcriptional machinery components and control the processing of viral genetic information via direct binding to nucleic acids or through binding to host transcriptional cofactors. The scheme shows how vTRs hijack cellular transcriptional components to transcribe the viral gene encoding vTR. Once synthesized and returned to the nucleus, vTR stimulates transcription of the same transcription unit (vTR itself) via a two-step autoregulatory loop (B) or a different viral protein X via cascade regulation (C). Red arrows depict the end of promoters (red regions) on the genome where vTRs bind. (D) Identified regions essential for vTR function may serve to design small molecules that specifically inhibit vTRs, suppress viral genome transcription and activate host cell protective pathways.

rectly via cofactors upon HTLV-1 infection – causing adult T-cell lymphoma; and (iii) Rta (replication and transcription activator) binding dsDNA within EBV – causing infectious mononucleosis along with lymphoproliferative diseases. The following sections describe particular roles and the most critical interacting partners of vTRs mediating three diverse oncoviral transcription strategies.

HBV: life cycle and strategy of viral genome transcription

HBV is a hepadnavirus containing a DNA genome. HBV causes acute damage to infected hepatocytes. The continuous release of viral particles (Figure 2A and B) evokes an immune response that promotes liver inflammation. The prolonged inflammation induces severe liver damage, insufficiency and an elevated risk of cirrhosis and tumorigenesis (22).

From the molecular point of view, the HBV genome comprises 3.2 kb of relaxed circular partially gapped dsDNA (rcDNA) (Figure 2C). One DNA strand is full length, but the complementary (+) DNA strand is incomplete (23). The transcription mechanism is mediated by host cell proteins that repair the viral (+) DNA strand and translocate rcDNA to the nucleus. Repaired, stable and entirely complementary DNA called covalently closed circular DNA (cccDNA) is transcriptionally active. The (-) strand of cccDNA is the transcription template for cellular RNA polymerase II to produce a longer than genome length RNA called the pregenome RNA (pgRNA) and shorter tran-

scripts, all of which serve as mRNAs (Figure 2C). The transcripts are exported to the cytosol for subsequent translation. The reverse transcription of pgRNA into the progeny DNA leads to the formation of rcDNA or the creation of linear dsDNA. The dsDNA mediates and significantly elevates the risk of HBV incorporation into the genome of hepatocytes (24,25). The shortest transcript encodes the HBx (Figure 2C). HBx is expressed early after the infection and rapidly promotes HBV genome transcription (26). HBx stimulates HBV transcription by activating cellular transcription pathways and affecting epigenetic signals on the cccDNA (27). HBV replication requires HBx, as confirmed in human hepatoma chimeric mice cells infected by HBV. In the experiment, HepaRG cells were infected with modified HBV expressing HBx under an inducible promoter. Other cells were infected by HBV only when HBx was expressed (28). Hence, HBx has been proven to be an essential regulator during the infection.

HBx structure and binding partners: transcription regulation roles

HBx is a 154-amino acid, 17-kDa protein. As presented in Figure 2D, the N-terminal part contains a phylogenetically conserved region rich in proline and serine residues (PSR). The PSR exerts autoregulatory activity via phosphorylation-based signaling and alters the subcellular localization of HBx (29,30). HBx enhances active HBV metabolism (cccDNA transcription) by subverting DNA

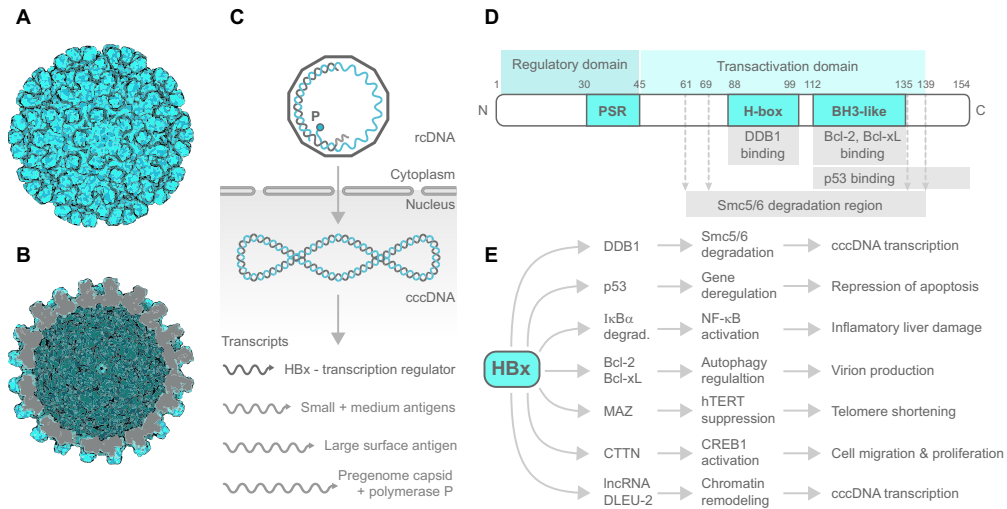


Figure 2. HBV capsid, viral DNA processing and HBx transcription factor domain structure, interactions and effects. The electron microscopy reconstruction of HBV capsid particle based on PDB 2G33: (A) outer view and (B) inner view, visualized using the Protein Imager. (C) Scheme of how HBV genome is processed in the host cell. Viral genome enters host cell as rcDNA (relaxed circular partially gapped dsDNA) containing viral reverse transcriptase (polymerase P, cyan circle) that converts rcDNA into dsDNA utilizing host RNA polymerase II and RNA primer at the 5' end of the gapped strand (light gray); cccDNA (covalently closed circular DNA). (D) Domain structure of viral transcription factor HBx. Numbers above denote position of amino acid; PSR, proline/serine-rich region; H-box, amino acid sequence interacting with cellular DDB1; BH3-like, the region comprising BH3-like motif and interacting with Bcl-xL protein family. (E) Overview of cellular proteins directly interacting with HBx along with immediate effects of interaction at the cellular level, and subsequent physiological response. Other HBx interacting partners are mentioned in the text.

repair-associated ubiquitin ligase machinery comprising cullin 4-DNA damage-binding protein 1 (CUL4-DDB1) (31). HBx employs the amino acid sequence of H-box (Figure 2D) that resembles the DWD motif present in DDB1 cullin-associated factors. The structurally conserved DWD motif is formed by a short α -helix (32). HBx through its helical H-box interacts with DDB1 directly (33,34). The primary function of the complex HBx-DDB1-E3 ligase is to target the Smc5/6 complex for degradation, through which HBx prevents the Smc5/6 complex from restricting viral cccDNA transcription (Figure 2E). Site-directed mutagenesis verified that amino acids C61, C69, C137 and H139 are substantial for Smc5/6 degradation effects of HBx, although they are not essential for DDB1 binding (34). Recently, Korniyev *et al.* showed that wild-type HBx is predominantly located in the nucleus of HBV-infected primary human hepatocytes. In contrast, an HBx mutant that could not bind DDB1 (33) was detected in both the cytoplasm and nucleus (35).

HBV strategy on the onset of infection toward enhanced survival of infected hepatocytes relies on suppressing endogenous metabolic stress elicited by the presence of viral transcripts and their products (36). HBx interacts directly with tumor suppressor p53 through a region comprising amino acids 112-136. Upon HBx binding, the DNA binding site on p53 remains unaltered, and HBx downregulates activation of transcription mediated by p53 (37). Hence, HBx affects p53 transcriptional activity but has no effect on DNA binding of p53.

Besides inhibiting several p53-mediated cellular processes that include DNA sequence-specific binding, transcriptional transactivation and apoptosis (38), HBx indirectly influences cell survival by affecting NF- κ B (nuclear factor of kappa B) pathways (39). NF- κ B is a member of the family of transcription factors that play an essential role in immune, inflammatory and apoptotic responses. HBx binds directly NF- κ B-associated inhibitor - I κ B α . HBx interacts with amino acids 249-253 of I κ B α . This region overlaps with the interaction site for p50 and p65 subunits of NF- κ B, and it is also near to the nuclear export signal sequence (40). Normally, I κ B α sequesters NF- κ B in the cytoplasm and inhibits NF- κ B function. When HBx-I κ B α form a complex, I κ B α is unable to bind and export NF- κ B from the nucleus, leading to NF- κ B activation. Consequently, the HBx-I κ B α complex attenuates NF- κ B-induced apoptosis that is mediated by targeting anti-apoptotic genes such as fas, c-FLIP and survivin, or genes from the Bcl-2 family (39). Moreover, Bcl-2 and Bcl-xL also belong to HBx interacting partners.

Recent structural data showed that cellular anti-apoptotic Bcl-xL binds HBx via the BH3 domain of HBx (41). Crystallographic data demonstrate that the BH3-like motif of HBx interacts with Bcl protein differently than other cellular proteins containing the BH3 domain. Interestingly, the HBV replicon comprising double mutant of HBx (W120A, L123A) failed to produce HBsAg and HBeAg in mice *in vivo*. *In vitro*, isothermal titration calorimetry measurements based on heat exchange recording during HBx titration to Bcl-xL confirmed that

the interaction between HBx and Bcl-xL is lost in the W120A, L123A HBx double mutant.

Furthermore, cell survival is promoted because HBx directly binds GRP78, a master regulator that triggers the stress response in the endoplasmic reticulum via the unfolded protein response system. HBx-GRP78 complex reduces ATF4, Bcl-2 and γ H2AX expression levels, promotes cell survival and downregulates DNA repair (42). Hence, GRP78 was suggested as a potential therapeutic target for suppressing tumorigenic processes (43).

Another hallmark of stress caused by HBx in tumorous hepatoma cells is the occurrence of shortened telomeres compared to adjacent hepatic tissue. HBx induces telomere shortening through binding the c-myc zing finger of MAZ. The complex HBx-MAZ binds preferentially to the telomerase promoter, suppresses its transcriptional activity and downregulates telomerase expression. Therefore, HBx works as a corepressor of MAZ that enhances telomerase repression (44).

HBx not only plays a vital role in promoting cell survival in HBV-positive cells, but also contributes to HBV-related tumorigenesis. HBx supports the stability of cortactin CTTN and transcription factor PAX8, two proteins accepted as tumor markers overexpressed in various cancers (45). Immunoprecipitation confirmed HBx-CTTN complex formation in HepG2 and metastatic hepatoma MHCC-LM3 cell lines. The CTTN levels correlate with increased levels of CREB1 as well as CREB1 downstream signaling toward tumor promotion and cell invasiveness. The complex HBx-CTTN restores upregulation of CREB1 signaling in CREB1 knockdown (46). The HBx colocalizes with the E2 ubiquitin ligase complex, where it interacts with Skp2. In the presence of the HBx-Skp2 complex, the ubiquitination omits PAX8 degradation. Consequently, the PAX8 level increases and mediates hepatocarcinoma development (45).

Besides the indirect influence on transcription via interaction with other transcription factors, HBx directly interacts with lncRNAs, including lncRNA DLEU2 (deleted in lymphocytic leukemia 2). As a result, HBx enhances DLEU2 transcription and its accumulation in HBV-infected cells and HBV-related hepatocellular carcinomas, serving as a tumorigenesis marker. HBx-DLEU2 interaction sites partially overlap with sites of another DLEU2 interacting partner – histone methyltransferase enhancer of zeste homolog 2 (EZH2), the catalytic subunit of the polycomb repressor complex 2 (PRC2), which belongs to the group of chromatin-modifying complexes. During HBV infection, HBx levels increase. If HBx:EZH2 ratio is 6:1 or higher, DLEU2 favors HBx binding. DLEU2-HBx co-recruitment on the cccDNA displaces EZH2 from the viral chromatin, supporting the transcriptionally active state of viral cccDNA and replication of the whole virus (47).

Implications and perspectives of HBx regulation in oncogenesis

Current HBV medication relies on the long-term HBV attenuation by a mixture of nucleoside/nucleotide analogs in combination with interferons and their pegylated derivatives, as reviewed recently (48). Entecavir and two deriva-

tives of tenofovir (disoproxil fumarate and alafenamide) are novel antivirals that display better resistance barriers in prolonged administration compared to older drugs. Another nucleotide analog candidate (ATI-2173) acts specifically as an HBV polymerase inhibitor and exhibits improved pharmacokinetics (49). Moreover, by acting in a noncompetitive manner, it is expected to effectively suppress HBV recurrence in combination with other nucleotide/nucleoside analogs.

A promising primary target for antiviral drug design among HBx-binding proteins might be DDB1, as HBx-DDB1 interaction was validated by structural studies (32). Importantly, HBx forms a complex with Cul4-DDB1 ubiquitin E3 ligase, where HBx acts as a 'substrate adaptor' via HBx-DDB1 interaction. HBx recruits critical cellular proteins for ubiquitination that ought to be removed to regulate host cell pathways successfully (50). Compromising the complex HBx-DDB1 by interfering molecules may significantly downregulate HBx activity and the whole HBV genome transcription. The attempts to find the inhibitors using a split luciferase assay system have been initiated (51).

Recently, other HBx structural studies identified a short oligopeptide comprising amino acids 118-127 that outcompetes the active motif of viral HBx from binding with Bcl-xL (41). Cellular metabolism impairment mediated via the BH3-like domain of the HBx oligopeptide was demonstrated *in vivo*. On this account, HBx oligopeptide presents a promising way to attenuate HBV infection (41). Coincidentally, the first synthetic BH3 mimetic (venetoclax) was approved by FDA as apoptosis-promoting drug in the treatment of chronic lymphocytic leukemia (52). The current research directed to the personalized profiling of BH3-mediated anti-apoptotic status of tumor cells could lead to the proper BH3 mimetic selection for an effective cure of difficult-to-treat cancers. Additionally, selective HBx oligopeptide treatment could prevent hepatocarcinoma promotion.

Similarly, HBV could be inhibited specifically on multiple levels by disrupting DLEU2-HBx interaction. The impairing of DLEU2 binding would silence cccDNA transcription. Moreover, preventing DLEU2-HBx association with target host promoters would maintain EZH2-mediated repression and lead to the transcriptional silencing of EZH2/PRC2 target genes that would have been regulated via HBx (47). Thus, finding a specific inhibitor disrupting DLEU2-HBx interaction would compromise both transcriptional and epigenetic activation at once.

The reviewed essential and recent findings underscore the importance of a deep understanding of interactions between HBx and cellular partners. The ongoing research exploring small molecule agonists and inhibitors shows encouraging results applicable in the treatment of especially chronic HBV patients with complications. As hepatocarcinoma is an aggressive cancer with high resistance to chemotherapy (53), the therapy based on the HBx-interfering approach is an attractive way to efficiently treat this type of cancer.

As HBx interacts with many host partners, HBV infection treatment based on HBx inhibition might require the combination of several specifically targeted drugs. Furthermore, the comprehensive HBx interface involved in protein-

protein interactions, together with utilizing shallow interaction pockets, might present complications in molecular research that could make HBx targeting challenging (54). Despite the possible structural and functional challenges connected to the unique HBx role in HBV infection, the invention of precise and effective HBx targeting deserves the full attention of the whole research community.

HTLV-1: life cycle and strategy of viral genome transcription

HTLV-1 is a delta RNA retrovirus that was first isolated in the early 1980s by two independent research groups (55,56). HTLV-1 belongs to the Retroviridae family, including the human immunodeficiency virus (HIV) (Figure 3A and B). HTLV-1 infects an estimated 15 million people worldwide annually (57). Although HTLV-1 infection is mostly asymptomatic, ~4% of carriers develop a severe malignancy of CD4⁺ T cells known as adult T-cell leukemia/lymphoma (ATL) after a latency period of three to six decades (58,59). HTLV-1 is also the causative agent of several inflammatory and immune-mediated diseases, most notably HTLV-1-associated myelopathy/tropical spastic paraparesis (60), and to a lesser extent HTLV-1 uveitis (61), infective dermatitis (62), myositis (63), arthritis (64) and bronchiectasis (65). ATLs are clinically classified into four distinct subtypes: acute, lymphomatous, chronic and smoldering. Acute and lymphomatous ATLs are highly aggressive with a median survival of <1 year, whereas chronic and smoldering represent more indolent forms of the disease (66,67).

Viral RNA processing in HTLV-1 is initiated by viral reverse transcriptase that mediates the synthesis of a dsDNA copy of the viral RNA genome. Viral DNA and integrase protein gain access to the nucleus mainly using intracellular trafficking machinery (Figure 3C). Viral integrase catalyzes viral DNA insertion into the host DNA (23). Transcription of integrated viral DNA (the provirus) by the host cell RNA polymerase II produces full-length RNA transcripts, which are used for multiple purposes: (i) full-length RNA molecules that are exported from the nucleus and serve as mRNAs to form Gag and Gag-Pol polyprotein precursors; (ii) parts of full-length RNA molecules become encapsidated as progeny viral genomes; and (iii) other full-length RNA molecules are spliced within the nucleus to form mRNA for the Env polyprotein precursor proteins (23).

HTLV-1 is transmitted mainly by breastfeeding (68), and to a lesser extent through sexual intercourse (69), and exposure to cell-containing infected blood components through transfusion or needle sharing (70,71). HTLV-1 transmission is mediated by cell-to-cell contact from infected to uninfected cells, not via free virions (72), and utilizes the activity of regulatory and accessory genes encoded by the 3' pX region (*tax*, *rex*, *p30*, *p12*, *p13* and *HBZ*) of HTLV-1 provirus (73). *Rex* has been shown to regulate post-transcriptional viral gene expression. Moreover, *Rex* increases viral RNA stability during the latency phase of the viral life cycle (74,75). The genes *p12*, *p13* and *p30* play significant roles in establishing and maintaining viral persistence (76). *HBZ* and *Tax* interact with host proteins to

alter their function and manipulate host cell signaling and transcriptional pathways, thus mediating HTLV-1 oncogenesis with successful evasion of the immune surveillance (77). *HBZ* is significant for the proliferation of infected cells, whereas *Tax* is crucial for viral replication and *de novo* infection. In contrast to the intermittent expression of *Tax* in ATL cells, *HBZ* is consistently expressed and antagonizes several *Tax* activities (77).

Tax plays an essential role in several mechanisms involved in leukemogenesis mediated by HTLV-1, including down- and upregulation of numerous microRNAs, differential mRNA expression, alternations in cell signaling, somatic mutations, and epigenetic deregulations or aneuploidy (78). *Tax* also activates several cell signaling pathways such as pathways depending on serum response factor, CREB/ATF (cAMP response element-binding protein/activating transcription factor family protein) and NF- κ B. Thus, *Tax* modulates viral and cellular gene expression (79,80).

Moreover, *Tax* affects the mechanisms controlling cell cycle progression, DNA damage response and apoptosis that allow *Tax* to favor the proliferation of infected cells and the genomic instability accumulation (78,80). Additionally, *Tax* has the potential to transform rodent fibroblasts (81) and induce cell transformation in transgenic mice (82). Unexpectedly, *Tax* has been reported to rarely transform human primary T cells, suggesting that other viral proteins rather cooperate to induce T-cell transformation and ATL development (83). For example, NF- κ B activation mediated by *Tax* represents a crucial step in the promotion of cell proliferation, which *HBZ* balances to enable the infected cells to escape immune surveillance (84).

Recently, Vandermeulen *et al.* analyzed the transcriptome and interactome of the *Tax* and *HBZ* proteins. They uncovered distinct but common host factors and RNA-binding proteins (such as U2AF2, a critical cellular regulator of pre-mRNA splicing) between *HBZ* and *Tax*. The authors found out that *Tax* and *HBZ* alter the splicing landscape in T cells. Interestingly, *Tax* favored exon inclusion, while *HBZ* induced exon exclusion. A subset of the splicing changes was also found in ATL patients and might represent clinically valuable biomarkers for ATL (85).

HBZ structure and binding partners: transcription regulation roles

HBZ is a 206-amino acid, 25-kDa protein. Figure 3D shows the predominant spliced transcript in ATL cells that is transcribed from a negative-sense RNA under the control of a 3' LTR promoter (73). *HBZ* possesses an N-terminal activation domain containing two LxxLL-like motifs that interact directly with the KIX domain of transcription coactivators CBP/p300 (Figure 3D and E). The KIX domain of CBP/p300 is also recognized by *Tax* (86). Once *HBZ* interacts with CBP or p300, *HBZ* interferes with *Tax*'s ability to bind CBP/p300. Thus, *HBZ* prevents CBP/p300 recruitment to the viral promoter and represses *Tax*-dependent HTLV-1 transcription activation. Moreover, *HBZ* interacts with other CBP/p300 domains, including both the HAT and C/H3 domains (87). Therefore, *HBZ* inhibits HAT ac-

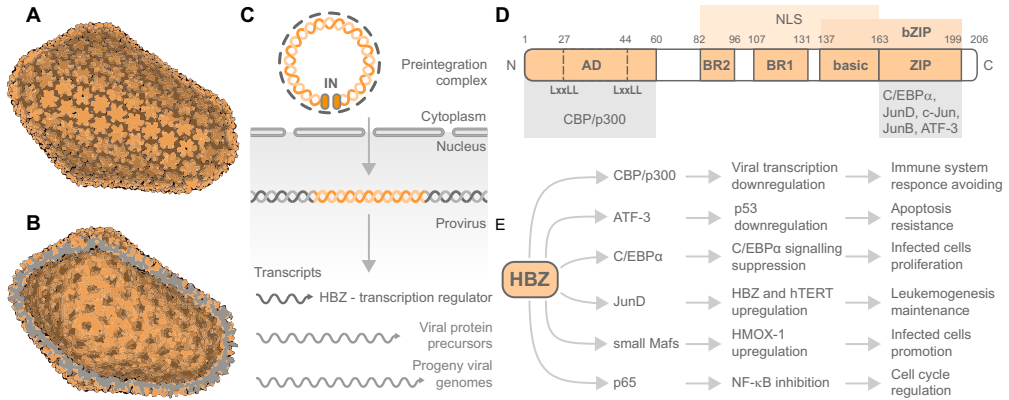


Figure 3. The retroviral capsid, viral RNA processing and HBZ transcription factor domain structure, interactions and effects. The electron microscopy reconstruction of HIV-1 capsid based on PDB 3J3Y: (A) outer view and (B) inner view, visualized using the Protein Imager. HTLV-1 and HIV-1 are close relatives within the Retroviridae family. (C) Viral RNA processing cycle of HTLV-1. The two copies of viral genomic RNA are reverse transcribed to form the pre-integration complex. The reverse-transcribed viral DNA and integrase (IN) enter the nucleus. The viral DNA incorporates into the host genome via integrative recombination catalyzed by IN. Thus, the created provirus is transcribed into mRNA. Expressed mRNA molecules produce viral proteins, including the transcription regulator HBZ and new viral genomic RNA. (D) Domain structure of viral transcription factor HBZ – the most abundant isoform. HBZ possesses an N-terminal activation domain (AD) containing two LxxLL motifs interacting with transcription coactivators CBP/p300. Central basic regions (BRs) of HBZ are responsible for nuclear localization. The C-terminal bZIP domain enables HBZ to heterodimerize with several bZIP factors. The bZIP domain consists of the basic region involved in DNA binding and a ZIP that mediates coiled-coil interactions with other bZIP transcription factors. (E) Overview of cellular proteins interacting with HBZ along with effects of the interactions on cellular functions and subsequent HTLV-1 progression and propagation. Other HBZ interacting partners are mentioned in the text.

tivity of p300/CBP, causing a reduction in p53 acetylation and repression of p53 activity (88). HBZ also contains three distinct regions in its central part that direct HBZ to the cell nucleus (Figure 3D): two nuclear localization signals (NLSs) correspond to basic regions containing the stretch of three to five positively charged residues, known to be present in several NLSs of nuclear proteins. The third basic region precedes a leucine zipper domain (ZIP) located at the C-terminal region of HBZ (Figure 3D). Together, the C-terminal regions form a bZIP domain that regulates the activity of several bZIP factors (Figure 3D and E). ZIP forms coiled-coil interactions with similar domains found in other bZIP transcription factors such as CREB, CREB-2, ATF1, ATF2, ATF3, C/EBPα and c-Jun. Consequently, heterodimerization between HBZ and these factors affects positively or negatively their transcription (Figure 1C).

Specifically, HBZ inhibits C/EBPα and c-Jun activity by sequestering them into transcriptionally inactive nuclear bodies, which supports the proliferation of ATL cells (89,90). Additionally, HBZ interacts with JunD (Figure 3D and E), a transcription factor, that is highly expressed in ATL cells. Heterodimerization of HBZ with JunD stimulates JunD expression from the viral 3' LTR and enhances transcription of cellular genes such as *hTERT*. Consequently, HBZ reactivates telomerase and therefore may contribute to the development and maintenance of the leukemic process (89,90). HBZ has also been reported to activate transcription of *HMOX1*, an oxidative stress response gene, via the interaction with small Maf proteins – transcription factors MafF, MafG and MafK (Figure 3D and E) (91). The increased levels of HMOX1 by HBZ protect

cells from oxidative stress. Therefore, HBZ supports ATL maintenance.

Due to constant HBZ expression in infected cells, HBZ is thought to be essential for the differentiation of infected cells into various hematopoietic cells. To enable HTLV-1 transmission and to evade host immune response, HBZ promotes transcription of *Foxp3*, *CCR4* and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (77).

Additionally, HBZ suppresses host classical NF-κB-driven transcription mediated by p65 (Figure 3E) by two mechanisms: (i) the inhibition of p65 DNA binding and (ii) the enhanced degradation of p65 through PDLIM2 E3 ubiquitin ligase expression. Moreover, HBZ represses transcription of some classical NF-κB target genes, including *IL8*, *IL2RA*, *IRF4*, *VCAM1* and *VEGFA* (92). A typical example of the NF-κB target gene that HBZ regulates is the cyclin D1 promoter gene. Cyclins control cyclin-dependent kinases and thus regulate the G1/S phase transition of the cell cycle. Ma *et al.* found that HBZ inhibits cyclin D1 mRNA level through binding with CREB, an action opposite to the G1/S transition induced by Tax (93). These findings suggest that the HBZ-mediated inhibition of NF-κB in cooperation with Tax-mediated activation is beneficial for infected cells' proliferation and oncogenesis.

Interestingly, not only HBZ protein but also HBZ RNA, which cannot produce the HBZ protein, is implicated in cell proliferation and ATL (94). HBZ RNA enhances transcription of the *survivin* gene, which counteracts apoptosis induction by HBZ protein. In this way, HBZ protein and HBZ RNA can oppose one another. The RNA-protein competition may present an elegant mechanism for controlling

the proliferation and survival of ATL cells and HTLV-1-infected cells.

Implications and perspectives of HBZ regulation in oncogenesis

ATL treatment is highly challenging due to the short median survival of patients diagnosed with aggressive forms (12). Moreover, HTLV-1 can avoid immune detection while maintaining or increasing the viral reservoir, which makes the treatment and prevention difficult and should be considered when developing future strategies (73).

The current treatment of ATL includes multi-agent chemotherapy (67) or allogeneic hematopoietic stem cell transplantation (95). Nowadays, patients with ATL lymphoma rely on chemotherapy with concurrent or sequential antiretroviral therapies consisting of zidovudine (ZDV) and IFN- α combination (96). Eventually, aggressive forms of ATL are treated by chemotherapy combined with humanized anti-CCR4 monoclonal antibodies (95). Intensive research in the last several years brought novel ATL treatment approaches, including pro-apoptotic molecules such as Bcl-2 inhibitors (95), cyclin-dependent kinase 9 inhibitors (97), and combined arsenic/interferon therapy with ZDV and IFN- α (98). In more detail, the promising mechanisms and drugs developed to inhibit ATL cell proliferation or to induce apoptosis have been recently reviewed elsewhere (78,99,100).

With millions affected worldwide, HTLV-1 is a significant problem in endemic communities. Remarkably, there are no effective vaccine or treatment options to prevent ATL so far. Current clinical treatments can slightly improve the overall patient survival (101). However, ATL patients' median survival is still <1 year. To help identify and develop novel effective therapies for ATL, we need a deeper understanding of molecular events leading to HTLV-1-induced oncogenicity. We expect that discovering both novel preventive or therapeutic strategies for HTLV-1 treatment might bring relief to infected individuals. Moreover, the new approaches could augment the understanding of other infections with oncoviruses.

As the HTLV-1-mediated oncogenesis is a cumulative result of multiple proteins, there can be several strategies used to prevent and treat ATL. Based on the successful entry inhibition of another retrovirus, the HIV (102), previous approaches to treat HTLV-1 treatment have focused on the entry steps of HTLV-1 replication cycle since binding of the virus to the cellular entry factors is required for viral transmission to non-infected cells. The strategies targeted envelope glycoprotein (Env) as a potential candidate for HTLV-1 vaccine and therapeutics (103). Simultaneous targeting of Env and accessory proteins including Tax and HBZ (80,104) could increase treatment efficiency and improve recovery prognosis.

The Tax sensitivity to host immunity and ubiquitous HBZ expression in ATL cells makes these viral proteins attractive therapeutic targets (105). For example, a complex and cooperative interplay between Tax and HBZ maintains the NF- κ B signaling equilibrium to drive clonal expansion balanced by senescence inhibition. The malignant transformation of infected T cells likely depends on the optimal level

of signal transduction fine-tuned by Tax and HBZ (80). It is critical to thoroughly elucidate the relation between Tax and HBZ for a deeper understanding of oncogenic mechanism mediated by HTLV-1.

There has been no vaccine preventing HTLV-1 confirmed in clinical trials yet. Recently, an anti-HTLV-1 lentiviral vector-based vaccine that encodes a unique polypeptide derived from Tax, HBZ, p12 and p30 accessory viral proteins has been developed. Experiments in mice have proven that the vaccination is safe and efficient inducing a cellular response that makes the vaccine promising for further studies (78). Additionally, Raza *et al.* carried out *in silico* studies that suggested the development of a vaccine against HTLV-1 vTRs. The authors predicted HBZ and Tax antigenicity and concluded that HBZ is a potential drug target. In contrast, Tax is a potential candidate for an epitope-based universal vaccine, which might activate the acquired immune system and stimulate the desired immune response against HTLV-1 (103). However, the vaccine potency should be confirmed via *in vitro* and *in vivo* immunological assays.

As HBZ performs an essential role in the proliferation of HTLV-1-infected cells, HBZ might provide a unique unrevealed mechanism that allows infected cells to evade immune recognition (106). One of the open questions is whether there is a direct connection between HBZ and cell energy deregulation, as the only missing cancer hallmark associated with HBZ. Direct HBZ connection with all essential cancer characteristics *in vivo* is yet to be confirmed to show a specific HBZ role in oncogenesis and to prove HBZ as an effective target for the development of new anti-HTLV-1 therapies.

Despite the growing evidence that HBZ is implicated in HTLV-1 pathogenesis, there is still little known about HBZ RNA functions. According to Gazon *et al.*, HBZ RNA inhibits sense transcription of HTLV-1 (107). They observed an RNA-dependent mechanism leading to a complete silencing of viral expression. Consequently, the induced viral expression silencing allows HTLV-1 entry into latency and escape from the immune response. Inhibition of HBZ RNA function in the mechanism could present a potential strategy for HTLV-1-associated disease treatment. Taken together, we suggest that a deeper understanding of HBZ functions might indicate new perspectives for the regulation of HTLV-1 progress and subsequent potential clinical applications.

EBV: life cycle and strategy of viral genome transcription

EBV is a ubiquitous human virus that belongs to the gamma-herpesvirus family. EBV was initially isolated from a cultured Burkitt's lymphoma (BL) cell line by Epstein *et al.* in 1964 (108). In 1984, EBV became the first fully sequenced herpesvirus with 172 kb genome encoding over 80 genes. The viral genome includes Epstein-Barr nuclear antigens (EBNAs) and latent membrane proteins (LMPs). The EBV genome persists in linear dsDNA form in viral particles (Figure 4A and B). DNA circularization is achieved by joining multiple 0.5 kb long terminal direct sequence repeats, creating the so-called episome (Figure 4C). The internal sequence repeats divide the viral genome into long

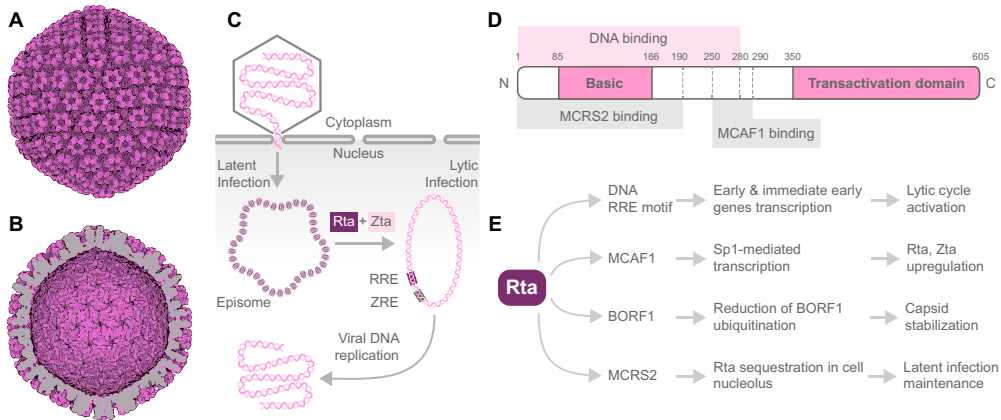


Figure 4. EBV capsid, viral DNA processing, and Rta transcription factor domain structure, interactions and effects. The electron microscopy reconstruction of EBV capsid particle based on PDB 7BSI: (A) outer view and (B) inner view, visualized using the Protein Imager. (C) Schematic representation of how EBV genome is processed in the host cell. Transcription factors Rta and Zta induce transformation of the latent to lytic infection of EBV. Rta and Zta bind to their response elements and converse the episomal viral DNA condensed on histones to the replication-ready circular dsDNA to produce viral DNA. RRE, Rta response element; ZRE, Zta response element. (D) Domain structure of viral transcription factor Rta. Rta contains the N-terminal basic and C-terminal transactivation domain. DNA binding region is localized within the 280 N-terminal amino acids. The Rta amino acid region 250-290 is essential for MCAF1 binding, important for Rta upregulation. The interaction with MCRS2 through Rta amino acid region 1-190 sequesters Rta in the nucleolus. (E) Overview of viral and cellular proteins interacting with Rta along with effects of the interactions on viral and cellular functions and subsequent EBV progression and propagation.

and short unique domain regions (109). EBV infects >90% of the human population during their lifetime, mostly without developing any symptoms (110). EBV spreads primarily through saliva. Studies have proven that EBV is a primary cause of infectious mononucleosis (111). Besides infectious mononucleosis, 200 000 cancer cases per year are also linked to EBV (112). Moreover, EBV is also associated with the development of autoimmune diseases such as multiple sclerosis (113).

EBV mainly infects two cell types: B lymphocytes and epithelial cells. Less commonly, EBV can infect natural killer T cells. EBV-related cancers differ based on the infected cell type (114). When EBV targets B lymphocytes, Hodgkin's lymphoma, BL or post-transplant lymphoproliferative disorder (PTLD) can develop. If EBV infects epithelial cells, EBV can cause nasopharyngeal carcinoma (NPC), gastric carcinoma (GC) or breast cancer (BC) (115).

EBV infection occurs in two states: latent and lytic (Figure 4C). EBV commonly infects B lymphocytes or epithelial cells in latent form and uses the host's polymerases to replicate during cell division. On one hand, a minimal number of viral genes are expressed in the latent state (116). On the other hand, Epstein-Barr encoded RNA, a small noncoding and non-polyadenylated RNA, is highly expressed in the latent state (117) and can be used as a marker of EBV infection (117,118). The latent state allows long-term survival of the EBV in human host cells where the viral genome persists as an episome in the nucleus (Figure 4C). The latent genes' expression can be involved in cancer development. For instance, EBNA-1, one of the latent genes, plays a role in BL, and LMP-1 is involved in NPC. The transition from latent to lytic state is called virus reactivation. *In vitro*, the

reactivation is triggered by 12-*O*-tetradecanoylphorbol-13-acetate, sodium butyrate, anti-Ig and TGF- β (transforming growth factor-beta). Nevertheless, the exact molecular mechanism that drives reactivation *in vivo* remains enigmatic (119).

It was shown that immediate early genes *BRLF1* and *BZLF1* that encode transcription factors Rta and Zta, respectively, play an essential role in virus reactivation (120). Rta and Zta upregulation is necessary for full expression of the EBV proteins, including viral DNA polymerases, during the immediate-early stage of the lytic cycle (121). Rta can also upregulate its own expression via a positive autoregulatory loop (Figure 1B) (122). Significantly, Zta closely cooperates with Rta to synergistically activate EBV early genes. Zta has been reported as the first transcriptional factor that preferentially binds to and activates the methylated *BRLF1* promoter (Rp). Zta binding to the methylated Rp, facilitated by serine residue 183, activates transcription of Rta (123,124). Thus, the binding of Zta to Rp can serve as a switching mechanism between the latent and lytic states of the EBV infection (Figure 4C).

Zta belongs to the family of basic leucine zipper (bZIP) transcription factors with a transactivation domain that was described to be essential for recognition and high-affinity binding to methylated DNA (125). Additionally, Zta can be secreted from the cell and directly penetrate the cell membrane using its cell-penetrating domain. In EBV-infected cells, Zta alone can switch EBV from latency to lytic cycle. Therefore, Zta may transfer reactivation signals between infected cells (126,127). During the latent stage of the virus cycle, the EBV virion genome becomes heavily methylated by the cellular DNA methylation machinery

(128). Zta binds methylated DNA and activates transcriptionally silent host genes (129,130). It was suggested that *BZLF1* gene expression could directly or indirectly contribute to EBV-induced tumorigenesis. Zta involvement in EBV-induced oncogenesis was recently reviewed comprehensively (131).

Rta structure and binding partners: transcription regulation roles

Rta is a 605-amino acid, 67-kDa protein encoded by the EBV gene *BRLF1* (Figure 4D). Histone acetylation at the *BRLF1* promoter allows Rta expression, which further leads to activation of the viral lytic cycle (132). Rta is one of the least structurally characterized vTRs. It was shown that Rta forms homodimers but not monomers in solution. DNA binding region of Rta is localized within the 280 N-terminal amino acids and the dimerization region within the 232 N-terminal amino acids. Furthermore, no direct homologies were identified compared to other known DNA binding or dimerization motifs. The C-terminal part of Rta contains a transactivation domain rich in proline and acidic residues (133).

Rta presents a vTR of EBV that directly binds to the Rta response element (RRE) and transactivates a series of lytic genes, including the viral lytic gene PAN (Figure 4D and E). Rta binding to RRE has been described qualitatively by EMSA (134). Moreover, the Rta mutant lacking the C-terminal part comprising the last 55 amino acids can still bind DNA and display binding activity to *BMLF1* promoter *in vitro* (135). Importantly, Rta can also form a complex with other proteins and regulate transcription via a mechanism independent of the RRE binding (136).

As mentioned before, the synergistic activity of Rta with Zta is a crucial factor in activating the series of early lytic genes. It was shown that Rta interacts with Zta through MCAF1 (MBD1-containing chromatin-associated factor 1) (Figure 4E). The assembled complex binds to Zta response element (ZRE) and synergistically promotes the transcription activation of the EBV lytic genes (137). MCAF1 plays a critical role in the AP-1-dependent Rta activation of *BZLF1* transcription due to MCAF1 complex formation with Rta and ATF2. Moreover, the Rta–MCAF1–ATF2 complex binds the *BMRF2* promoter, a critical viral gene for EBV infection (138). The Rta amino acid region 255–290 is essential for MCAF1 binding (Figure 4D). MCAF1 also mediates Rta interaction with Sp1-interacting protein, where Rta increases the Sp1-mediated transcription, leading to the upregulation of Rta and Zta (139).

Recently, it was found that Rta is stably expressed in B lymphocytes during EBV latency and is present mainly in the nucleolus. The nucleolar sequestration of Rta is regulated by the interaction with MCRS2 protein (Figure 4D and E) and impacts the EBV lytic progression by inhibiting the Rta transactivation activity. Additionally, Rta is associated with ribosomes and enhances cellular translation in the nucleolus (140).

Another essential feature of Rta is initiating a cellular senescence program in epithelial cells and promoting the au-

tophagy induction via ERK1/2 activation in the early lytic phase. Taken together, Rta can stimulate growth arrest, induce senescence and modulate autophagy in infected cells (36,122).

Despite the Rta function as a regulator of host factors, it can also interact with viral proteins. Specifically, the Rta is present on the EBV capsid, where it directly interacts with EBV capsid protein BORF1 (Figure 4E). Rta reduces ubiquitination and thus stabilizes BORF1. Hence, Rta acts as an EBV tegument protein that stabilizes EBV viral capsids (139).

Implications and perspectives of Rta regulation in oncogenesis

EBV-related oncogenic diseases are often fast-growing tumors where early detection and immediate treatment are crucial for successful recovery. Regardless of the relatively high cure rate of B lymphocyte malignancies in developed countries, the cure rate remains low in less developed countries where health care is less available. In contrast, epithelial cell malignancies such as NPC, GC or BC have overall high mortality. Mechanisms of EBV-related oncogenesis have been already thoroughly reviewed elsewhere (141–144).

Despite the progress that has been made in the comprehension of the EBV connections to cancers, several aspects of EBV-related oncogenesis are still unknown and represent a major challenge in cancer research. Thus, new and potentially less demanding treatments, not involving chemotherapy and radiotherapy, that target EBV latent genes expression and vTRs could increase treatment efficiency and improve recovery prognosis (141,142).

EBV is associated with several malignancies, including PTLT. PTLT treatment consists of rituximab and immunosuppression reduction together with chemotherapy (145). Cellular therapy utilizing EBV-specific cytotoxic T lymphocytes provides an alternative to the conventional treatments of PTLT (146). Another strongly driven malignancy by EBV is NPC. The EBV protein LMP-1 was identified as a potential therapeutic target against EBV-positive NPC. LMP-1 promotes tumorigenesis by affecting various oncogenic pathways (147). Recently, DNazyme (DZ1) was designed to target the LMP-1 mRNA specifically. DZ1-based treatment could reverse the malignancy and promote the radiosensitivity of NPC (148).

The lytic form of EBV can be effectively inhibited by the guanine nucleoside analogs acyclovir and ganciclovir. They both inhibit viral DNA replication by silencing the viral DNA polymerase during the lytic cycle (149,150).

The majority of EBV-associated diseases are indirect results of viral replication. Moreover, EBV-associated malignancies are caused by the expression of viral genes that function as survival signals keeping EBV in a latent state. Therefore, inhibiting viral replication is ineffective in treating EBV-associated malignancies or infectious mononucleosis. As an alternative approach, the lytic cycle induction has been proposed as a therapy. Adenovirus vectors expressing Rta and Zta can be used to stimulate viral replication via lytic cycle activation (151), thus sensitizing EBV-infected tumor cells to nucleoside analogs. Combined treat-

ments using analogs and chemotherapy are more effective than monotherapy (152,153). Even though the lytic cycle can inhibit tumorigenesis due to the final lysis of the infected cells, several lines of evidence suggest that lytic cycle initiation contributes to tumor progression (143,154). Since Rta and Zta induce the lytic cycle in cooperation with other viral and cellular proteins, Rta and Zta could be employed to prevent EBV-driven oncogenesis after lytic cycle initiation. For example, targeting EBV replication by antirheumatic drug leflunomide inhibits the earliest step of lytic EBV activation – *BZLF1* and *BMRF1* expression and prevents the progress of EBV-induced lymphomas (155).

The complex interactions between the EBV and their hosts have made it challenging to design useful vaccine strategies to protect against EBV-associated diseases (156), and despite more than four decades of development, no licensed prophylactic vaccine against EBV is available (157).

The research has mainly focused on the EBV membrane antigen containing glycoprotein gp350, the uppermost abundant glycoprotein on the surface of virions and the most expressed by cells infected with EBV, as the most suitable vaccine candidate (157). The gp350 vaccine reduces the incidence of infectious mononucleosis. However, in phase II of clinical trials, the vaccine failed to prevent EBV infection. Moreover, designing a live-attenuated herpesvirus vaccine is improbable due to the persistence in infected individuals.

According to the challenges in current EBV treatment, the viral transcription factors present a new potential strategy for therapy. Here, Rta and Zta have been reviewed as essential and indispensable parts of EBV lytic cycle reactivation. Most of the EBV-associated diseases and malignancies develop in the latent viral state. The activation of the lytic cycle sensitizes infected cells to antiviral medication and may prevent the progression of EBV-associated diseases. Hence, the upregulation of Rta presents a promising target for lytic cycle induction as an alternative EBV treatment.

We can hypothesize several approaches for Rta upregulation that could lead to EBV lytic cycle activation: (i) inhibit the Rta–MCRS2 interaction, which could prevent Rta sequestration in the nucleolus and promote the Rta transactivation activity; (ii) design agents that could specifically bind to BRLF1 promoter and activate the Rta translation; (iii) employ adenovirus vectors expressing BRLF1; or (iv) a combination of mentioned strategies. Another approach how to suppress EBV tumors can employ the cytotoxic T lymphocyte's response to Rta and Zta. Further studies of Rta and its viral transcriptional activity are needed for a deeper understanding of how vTRs could be used for effective EBV treatment.

RELEVANCE AND PROSPECTS

The complete eradication of virus-induced diseases remains beyond the reach of current therapies despite significant advances in the development of effective vaccines. A surprising challenge is convincing the population that the vaccination is necessary and beneficial, as shown during the COVID-19 pandemic. When the part of the population refuses vaccina-

tion, finding the proper antiviral treatment for patients with severe disease outbreaks is even more critical. Thus, the demand for antiviral treatment that might selectively eliminate viral proteins and their activity is urgent.

For the successful spread of infection, viruses have evolved to sneak through the innate and adaptive antiviral response at both the cellular and whole organism levels. The current extensive studies of the life cycle of viruses brought novel molecular and structural knowledge of infection mechanisms. Nevertheless, little is still known about the functions of vTRs that enable viral genes to express and affect host gene expression. As vTRs are critical for effective viral infection, they are central to human disease pathogenesis and attractive targets for therapy.

Nowadays, the emphasis is placed on designing the pan-antiviral agents that could be utilized as a broad-spectrum viral treatment. Here, vTRs present a potential pan-antiviral target, affecting and modulating the host gene signaling pathways. vTRs directly bind specific DNA motifs (Rta and Zta) or protein domains (HBx and HBZ) (18). We can employ a transcription factor decoy (TFD) strategy to design oligonucleotides and oligopeptides to saturate and inactivate the binding sites of vTRs.

Targeting vTRs inside the cell is significantly more problematic as interfering molecules (Figure 1D) have to pass through cell and nuclear membranes. This limit could be diminished by the application of sophisticated drug delivery ways such as improved lipid-based nanoparticles that can deliver a therapeutic agent into the cell and biodegradable vectors designed to deliver mRNA coding protein parts for TFD to the cytoplasm (158). The second challenge is the limited use of antibody-based therapy against vTRs that is connected with the location of vTRs in the nucleus. This challenge can be partially overcome for vTRs that induce antigens on the cell surface. The third challenge is connected with the wide functional and interactional diversity of vTRs. The binding variety of vTRs might bring an interaction detour that would overpass a drug-inhibited interaction. Inhibition of more interacting domains of vTRs would minimize the interaction bypass possibility. The final problem is the long latency time for oncoviruses HTLV-1 and EBV. One of the most promising solutions might be controlled induction of transition from latent to lytic state with subsequent immunotherapy.

The previously mentioned limitations and challenges of vTR targeted treatment are prevailed by high specificity of the therapy focused on viral proteins with expected minimal effects on host cell proteins. In parallel, additional studies of vTRs and their interaction partners should be carried out to identify critical sites for disturbing vTR complexes that have been reviewed here. We suggest that vTRs should be examined by combining advanced methodological approaches *in vitro* and *in vivo* to reveal critical turning points that could be utilized for specifically targeted interference therapy.

The question of how vTRs' binding to human transcriptional cofactors in host cells affect functions of the human proteins is essential regarding the possible side effects and acquired resistance of inhibiting vTRs. Additionally, what are the most specific and reliable ways of targeting vTRs in relevant cell types? Another cardinal question is what the fate of vTR complexes would be after disabling critical in-

teractions and how it would affect host cells' response to viral genome expression. Finally, could vTR complexes be disturbed effectively by small molecules and thus used for specific eradication of viral infection? The lack of answers to the questions outlined above hampers the struggle with finding the way to defeat the most common human viruses. Elucidating the mechanism of vTRs' interaction might shed new light on virology and cell biology and reveal new, more successful antiviral-anticancer therapies. Eventually, revealing the Achilles heel in vTR action will help to develop a new effective treatment for virus-induced diseases against which vaccination strategy could not be applied.

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