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PROBLEMS & PARADIGMS

Prospects & Overviews

The multifaceted h*TR* telomerase RNA from a structural perspective

Distinct domains of h*TR* differentially interact with protein partners to orchestrate its telomerase-independent functions

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Abstract

Human telomerase progressively emerged as a multifaceted ribonucleoprotein complex with additional functions beyond telomeric repeat synthesis. Both the hTERT catalytic subunit and the h*TR* long non-coding RNA (lncRNA) subunit are engaged in highly regulated cellular pathways that, together, contribute to cell fitness and protection against apoptosis. We recently described a new role for h*TR* in regulating the abundance of replication protein A at telomeres, adding to the growing repertoire of h*TR*'s functions. Here, we focus on the non-canonical roles of h*TR* and discuss them in the context of the structural elements of the lncRNA. We propose that some functions of h*TR* may compete amongst each other through distinct interactions with its partners, proteins or mRNAs. We postulate that h*TR*'s non-canonical functions may be highly relevant in the context of normal somatic cells that naturally silence h*TERT* gene, while keeping h*TR* expression.

KEYWORDS
lncRNA, non-canonical functions, telomerase RNA

INTRODUCTION

Mammalian telomeres are tandem repeats of DNA sequences and associated proteins forming specialized ribonucleoprotein (RNP) structures at the ends of chromosomes. Telomeres prevent the chromosome ends from being recognized as DNA double-stranded breaks by the cellular DNA damage response (DDR) machinery. In human somatic cells, the telomeres shorten progressively with each cell division, due to the "end replication problem" arising primarily from the incomplete lagging strand synthesis and nucleolytic processing of leading strand.[1,2] This progressive telomeric erosion is overcome by the action of the specialized RNP enzyme called telomerase. Human telomerase is a bi-lobed structure comprising two major components: a catalytic reverse transcriptase subunit (hTERT) that adds short repetitive DNA sequences, using the second component—h*TR*, a long non-coding RNA component—as the template for telomeric repeat synthesis. The h*TR* RNA, in turn, binds two sets of the four mature H/ACA RNPs—Dyskerin (DKC1), GAR1, NHP2, and NOP10—for formation of a functional telomerase holoenzyme complex[3–6] (Figure 1A).

Telomerase activity is developmentally regulated and absent from normal somatic cells. Consequently, progressive telomere shortening renders them critically short, dysfunctional, and thus eliciting a DDR that triggers cellular senescence.[7] Telomerase inactivation, in normal somatic cells, is correlated with h*TERT* gene silencing, as cells naturally continue to express significant levels of h*TR* RNA.[8] Somatic cells with oncogenic gains can reactivate h*TERT* to maintain their telomere lengths and reach immortality. Reactivation of telomerase enzyme is observed in 85%–90% of cancer cells (TEL^+ cells), while 10%–15% of them lack telomerase activity and instead hijack a subset of cellular DNA replication and repair factors to perform "Alternative Lengthening of Telomeres" (ALT^+ cells).[8,9]

Abbreviations: ALT, alternative lengthening of telomeres; DDR, DNA damage response; lncRNA, non-coding RNA; RNPs, ribonucleoproteins

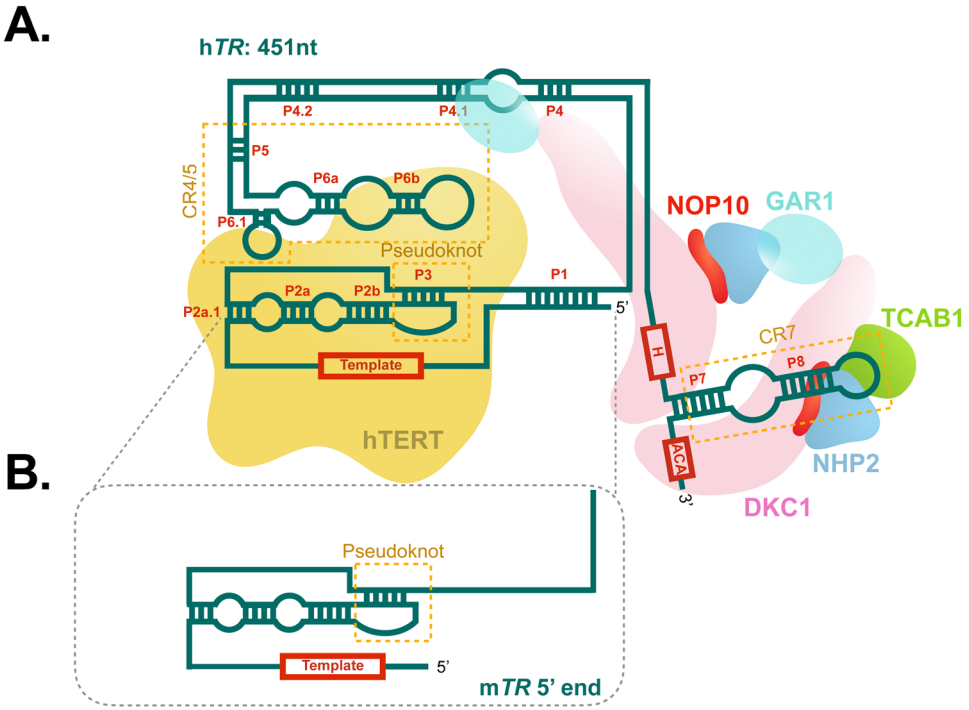

FIGURE 1 Structural features of telomerase RNA. (A) Secondary structure of h*TR* RNA in complex with hTERT and other accessory proteins to make up active telomerase holoenzyme through the indicated secondary helices. Based on recent cryo-EM structure of human telomerase complex,[3] h*TR* associates with one molecule of h*TERT* and TCAB1, along with two complexes, each comprising four molecules: Dyskerin, GAR1, NHP2, and NOP10. (B) 5′ end of mouse *TR* (m*TR*), compared to 5′ h*TR*. m*TR* lacks the P1 helix present in the human ortholog and predicted to be important for interactions with apoptosis-regulating mRNAs

Over the years, telomerase progressively emerged as a fascinating protein complex with additional functions beyond just maintaining telomeres and conferring replicative immortality. For instance, the catalytic subunit hTERT can act as a transcription (co-)factor to regulate the Wnt/β-catenin[10] and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways.[11] Loss of hTERT expression has also been postulated to compromise the DDR pathway, potentially through an alteration of chromatin structure, independently of its role at telomeres[12] Mitochondrial hTERT protein has further been implicated in non-canonical functions, as circular mitochondrial DNA lacks telomeres. However, the exact mitochondrial functions of hTERT remain controversial, with some studies suggesting anti-oxidative functions whereas others implying hTERT-induced oxidative stress.[13]

Similarly, the telomerase RNA subunit h*TR* has reportedly telomerase-independent functions, although most of them remain mechanistically poorly understood. Notably, h*TR* promotes cell survival by downregulating apoptosis in CD4$^+$ lymphocytes and cancer cells.[14–18] A recent report also showed that h*TR* can interact with several mRNAs that code for apoptosis-regulating proteins, suggesting potential RNA-RNA interactions-based extra-telomeric functions.[19] h*TR* has also been reported to regulate DDR by downregulating the ATR-mediated DDR.[17] After import into the mitochondria, where h*TR* is processed into a shorter form, and its export back to the cytosol, recent studies suggested a possible role for the shorter h*TR* in regulating cellular senescence independently of telomerase.[20,21] Finally, we have shown that h*TR* expression in ALT$^+$ cells can downregulate replication protein A (RPA) abundance at telomeres by promoting the catalytic subunit of DNA-PK (DNA-PKcs)- and hnRNPA1-dependent removal of the single-stranded DNA-binding protein, a process that likely promotes protection of telomeres 1 (POT1)-dependent telomere capping.[18] Together, our observations led us to propose that h*TR* may contribute to telomere capping through two distinct and independent mechanisms: a canonical, hTERT-dependent one and a non-canonical, DNA-PKcs/hnRNPA1-dependent one. Here, we will discuss these non-canonical functions of h*TR* in light of the essential structural elements of the lncRNA. We will also elaborate on the possible competition between the various functions of h*TR* through its predicted interaction with distinct protein or RNA partners.

OVERVIEW OF h*TR* STRUCTURE

Before diving into h*TR*'s non-canonical functions, it is imperative to understand the secondary structural features of h*TR*, as determined by several years of dedicated mutational analyses, analyses of telomeropathy-associated mutations, structure-based modeling, and advanced biochemical studies.

Mature h*TR* is a 451 nt-long RNA composed of four key conserved structural domains: the pseudoknot/template domain, the conserved regions 4 and 5 (CR4/CR5); the box H/ACA; and the CR7[3,4,22] (Figure 1A). The 5′ end of h*TR* comprises the P1 helix, followed by the

pseudoknot and the CR4/CR5 motives that, together, form the catalytic core domain that interacts with hTERT catalytic subunit for the telomerase activity (Figure 1A). The CR4/CR5 regions include a branched junction of RNA helices P5 and P6, and the activity-critical stem-loop P6.1.[3] Based on two recent cryo-EM structures, one molecule of h*TR* binds two sets of H/ACA heterotetramer proteins (GAR1, NHP2, NOP10, and Dyskerin).[3,4] The first set of H/ACA proteins contacts the hairpin P4 stem exclusively via Dyskerin, while the second set interacts more extensively with the 3′ hairpin P7 stem and CR7 stem-loops. P7 stem loop interacts primarily with Dyskerin but CR7 extends beyond to interact with NOP10, NHP2, and one molecule of TCAB1 (Figure 1A).

hTR-MEDIATED PROTECTION AGAINST APOPTOSIS

First reported in 2004 by the group of E. Blackburn as a telomere length- and p53-independent process,[14] the non-canonical, cell-protective, function of h*TR* in downregulating the apoptotic pathways was rapidly confirmed by others in a variety of cancer cell lines and immortalized cells.[15–18] Gazzinga and Blackburn's follow-up study, in 2014, further showed that this non-canonical function of h*TR* in protecting against apoptosis was also relevant for normal human $CD4^+$ T cells.[16] Reducing endogenous h*TR* using shRNA knockdown was found to activate the intrinsic apoptotic pathway characterized by increased levels of Puma protein, Bim upregulation and caspase-9 activation, albeit through a p53-independent and DNA damage-independent pathway.[16] However, in 2006, Kedde et al. reported a partial dependency on p53 for h*TR* knockdown-induced apoptosis and further reported that knocking down h*TR*, particularly when cells are subject to UV rays, leads to ATR kinase activity upregulation.[17] This upregulation in ATR kinase activity, they proposed, can stimulate phosphorylation of p53 and CHK1, thereby potentially culminating in the inhibition of cellular proliferation.[17] However, we recently reported that, under unperturbed conditions, h*TR* knockdown does not have an impact on global CHK1 phosphorylation, neither in TEL^+ nor in ALT^+ cells.[18] Our data indicate instead that h*TR* knockdown, while indeed associated with an extreme reduction in cellular viability in both TEL^+ and ALT^+ cell lines, only slightly affects ATR activation in ALT^+ cells, but not in TEL^+ cells, and this through a specific modulation of ALT telomeric RPA levels (see below), without any detectable impact on global CHK1 activation. Altogether, these new findings suggest an alternative—but poorly understood—mechanism, independent of h*TR*'s impact on ATR activity, to be responsible for protection against apoptosis.

In seeming contradiction to the studies in human cells that suggest an anti-apoptotic role for h*TR*, viable m*TR* knockout mouse models have been successfully generated, with no obvious deleterious phenotype in the first generations.[23] This could be explained by the fact that RNA interference (used in human studies) causes rapid reduction in h*TR* levels, whereas knocking-out m*TR* in germ line cells may allow for acquiring gradual mutations or compensatory events. Alternatively, the difference between mouse and human may stem from distinct non-canonical functions of *TR* in these two species, possibly due to differences in specific secondary structures of the respective non-coding RNA. In this regard, the secondary structure of the telomerase RNAs is highly conserved between human and mouse, except for the P1 helix at the 5′ end of h*TR*, which is absent from m*TR* (Figure 1B).[24]

Although the P1 helix of h*TR* is not in direct contact with the catalytic component of telomerase and is not required for high levels of telomerase activity,[3] this domain may be very close to hTERT (Figure 1A). Thus, we anticipate that the putative interaction of h*TR* with a subset of its partners, through the P1 helix, may be impaired when hTERT is bound to h*TR*. Consequently, h*TR* may be involved in protection against apoptosis only when hTERT is not bound. In line with this hypothesis, hTERT overexpression reduces the h*TR*'s ability to protect cells against apoptosis, suggesting that hTERT binding indeed competes with the anti-apoptotic functions of h*TR*.[16] Reinforcing this idea, the pathogenic G305A h*TR* point mutant—located in the P6.1 stem loop that completely abolishes catalytic activity and significantly reduces binding to h*TERT*—is proficient in protecting $CD4^+$ T cells from dexamethasone-induced apoptosis.[16] A recent study looking at the RNA interactome of h*TR*, with the idea that h*TR* might directly interact with mRNA molecules to act as a pseudouridylation guide and modify target mRNAs, brought additional evidences in favor of a competition model between hTERT and mRNA partners of h*TR*. Indeed, while 80 h*TR*/RNA interactions were detected in VA13 $hTERT^-/hTR^+$ cells, only 16 RNAs were found to bind h*TR* in HeLa $hTERT^+/hTR^+$ cells.[19] Intriguingly, 35 out of the 77 h*TR*-interacting mRNAs (three h*TR*-interacting RNAs are ncRNAs) code for proteins involved in cytoskeleton organization and/or the regulation of apoptosis—such as translationally controlled tumor protein (TCTP/TPT1) and filamin A (FLNA) mRNAs—suggesting an intricate RNA-RNA interaction network between h*TR* expression and cell survival.[19] More interesting is the fact that most of these interactions were predicted to involve the first 40 nucleotides of h*TR* 5′ end[19] that are exactly those engaged into the P1 helix and lacking from the m*TR* sequence (Figure 1B). Future studies will be necessary to examine whether there are differences between h*TR* and m*TR*'s interaction partners—RNAs or proteins—which may help to explain the differential roles of *TR* in the respective cell type. Also, important to mention here is the fact that, even though hTERT may compete with this non-canonical function of h*TR* in protecting against apoptosis, the lncRNA is known to be expressed in excess over the total telomerase RNP complexes,[25] suggesting the presence of an hTERT-free h*TR* pool in cells. In addition to a possible protective role against apoptosis for the 5′ end of h*TR*, the first 52 nucleotides of the telomerase RNA appear to be required for mitochondrial h*TR* import and processing into a shorter form of 195 nt in length that is exported back to the cytosol.[20]

h*TR*, DNA-PK, AND hnRNPA1: A ROLE IN THE RPA-TO-POT1 SWITCH AT TELOMERES?

As outlined above, h*TR* interacts with several accessory chaperone proteins through its H/ACA box and CR7 domain. These interactions

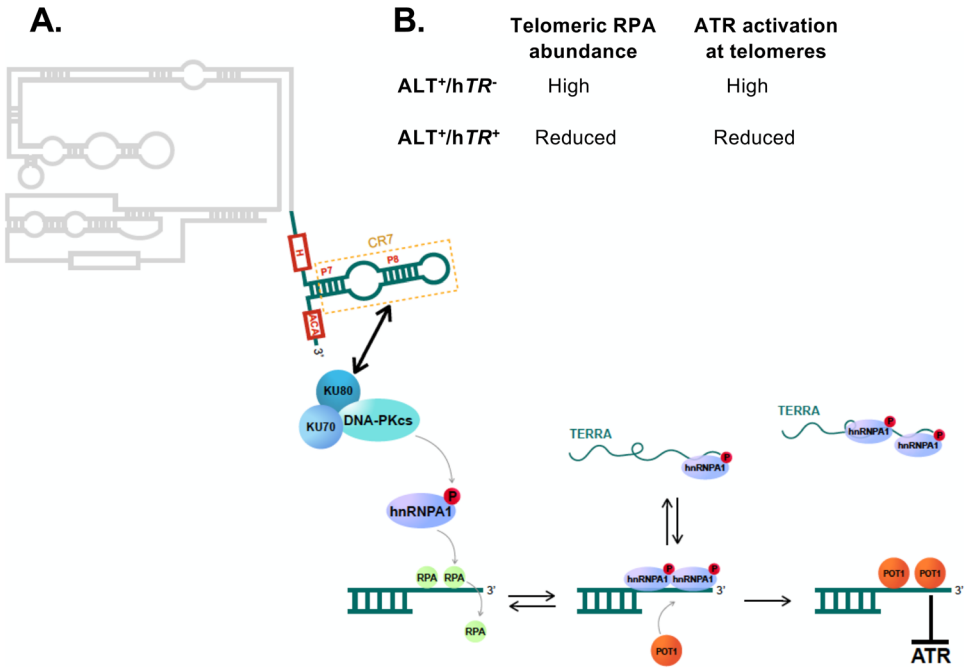

FIGURE 2 Proposed mechanism for how hTR regulates telomere end capping. (A) The CR7 motif in the 3′ end of h*TR* interacts with KU70/80 heterodimer to regulate the phosphorylation activity of DNA-PKcs towards hnRNPA1. Phosphorylation of hnRNPA1 increases its affinity for single-stranded telomeric DNA, thereby displacing RPA from telomeric ends. Subsequently, hnRNPA1 is stripped and sponged off the telomeres by TERRA to allow for POT1 binding. POT1 binding, in turn, ensures telomere capping and suppresses ATR kinase activity-mediated DNA damage response cascade at telomeres. Adapted from ref. 32. (B) Summary of the observed RPA abundance and ATR activation at telomeres of ALT$^+$ cells expressing or not h*TR*[18]

are essential for h*TR* biogenesis, processing, trafficking, and assembly into a functional telomerase complex[26] (Figure 1A). Importantly, the CR7 domain of h*TR* has also been involved in the interaction with the KU70/KU80 heterodimer. Originally identified in yeast,[27] the interaction between KU and h*TR* is conserved in humans, both in vitro and in cells, independently of hTERT. Minimally, KU70/80 heterodimer can interact with the 47 nt-long region of the CR7 motif in the 3′ end of h*TR*[28] (Figure 2A), but whether the binding to KU70/80 and H/ACA heterotetramer proteins is mutually exclusive in cells is still unknown. KU70/80, in turn, binds the DNA-PKcs to form the functional holoenzyme complex, important for DNA repair and with important functions in end-capping and maintenance of telomere length.[29] The interaction between h*TR* and KU70/80 has been observed in cells that use either telomerase or ALT mechanism, strongly suggesting that this interaction may have roles beyond telomerase-dependent telomere length maintenance.[28,30] Subsequently, hnRNPA1 protein—with important roles in telomere biogenesis—was identified as a direct substrate of DNA-PK, with the phosphorylation being stimulated by h*TR*.[30] In a separate study, not looking at h*TR*, DNA-PKcs-dependent hnRNPA1 phosphorylation was found to be critical for maintaining newly replicated telomeric 3′ overhangs by facilitating the switch from RPA to POT1 at these overhangs,[31] a process first reported by Flynn et al. that involves the TERRA telomeric non-coding RNA[32] (Figure 2A). This RPA-to-POT1 switch is critical to prevent hyperactivation of ATR-mediated DDR activation at the telomeric overhangs and to allow for protective telomere capping after replication.[32]

Put together, these data, obtained from cells naturally expressing h*TR*, suggested that, through its interaction with the DNA-PK complex, h*TR* may play a key role in facilitating the hnRNPA1-dependent RPA-to-POT1 switch at telomeres.

We recently brought evidence in favor of this hypothesis that h*TR* may facilitate an RPA-to-POT1 switch at telomeres by showing that the ectopic overexpression of h*TR* in ALT$^+$ cells—initially repressing h*TR* gene expression—downregulates telomeric RPA abundance. This, in turn, can reduce the ATR signaling activation at telomeres[18] (Figure 2B). This reduction in telomeric RPA abundance of h*TR*-overexpressing ALT$^+$ cells could be rescued by either hnRNPA1 depletion or DNA-PKcs inhibition, strongly suggesting that, in the presence of h*TR*, increased DNA-PK-mediated hnRNPA1 phosphorylation drives the h*TR*/hnRNPA1/DNA-PK complex into an "RPA-evicting" complex (Figure 2A). As the formation of this functional "RPA-evicting complex" is mediated by physical interactions between h*TR* and KU, future studies that map the interacting amino acids on KU, along with mutational studies that ablate the interaction, will be insightful. Such biochemical analyses will aid testing the hypothesis that h*TR* positively regulates the telomeric RPA-to-POT1 switch.

In the non h*TR*-expressing ALT$^+$ cells, hnRNPA1 may not be phosphorylated by DNA-PK, preventing it from catalyzing the RPA-to-POT1 switch and resulting in an increased abundance of RPA at single-stranded telomeric DNA, as we recently reported.[18] In the context of non h*TR*-expressing ALT$^+$ cells, we therefore predicted hnRNPA1 depletion or DNA-PKcs inhibition to be inconsequential

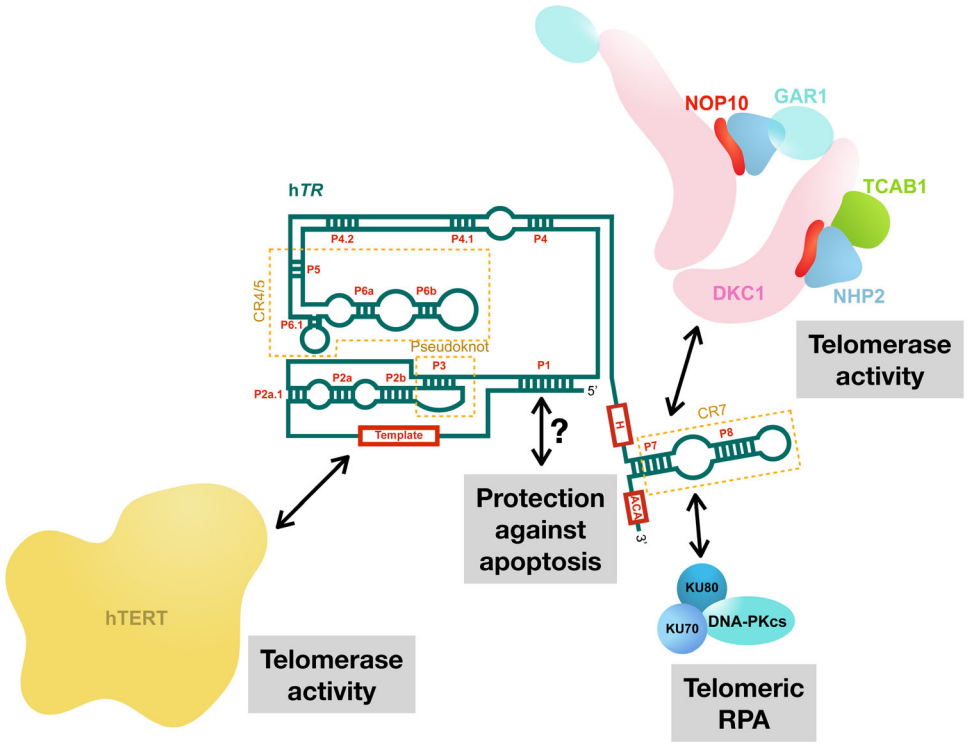

FIGURE 3 Hypotheses for non-canonical hTR functions through its distinct structural features. The interaction partners of h*TR* may direct its various functions. The 3′ CR7 domain of h*TR* contains overlapping binding sequences for KU70/80/DNA-PKcs or NHP2 protein. Potentially, h*TR* binds to either KU70/80 or NHP2, directing it towards its hTERT-independent or -dependent function in telomere capping, respectively. Finally, recent studies predict that the anti-apoptotic function of h*TR* may be regulated by P1 helix-mediated interactions with other mRNAs active within the intrinsic apoptotic pathway

to telomeric RPA abundance. Surprisingly, however, the ALT$^+$ cells lacking h*TR* expression showed a downregulation of telomeric RPA abundance following hnRNPA1 depletion or DNA-PKcs inhibition, suggesting that, in the absence of h*TR*, DNA-PKcs and hnRNPA1 may display RPA-promoting activity at telomeres. Altogether, our data therefore strongly suggest that h*TR* may act as a molecular switch that turns the DNA-PKcs/hnRNPA1 complex from "RPA-promoting" to "RPA-evicting," at telomeres.[18] By extension, we speculate that, in normal somatic cells (that constitutively express h*TR*), losing h*TR* completely may disrupt the RPA-to-POT1 switch at telomeres, leading to aberrant DDR machinery activation and thus telomere dysfunction. This could be one explanation to why most somatic cells typically retain h*TR* and silence h*TERT* gene.[33]

Our data from ALT$^+$ cells demonstrate that, while extremely low levels of h*TR* RNA are sufficient for its anti-apoptotic functions, higher h*TR* levels appear to be required for regulating telomeric RPA abundance.[18] Protection against apoptosis may not therefore sufficiently account for the constitutive maintenance of h*TR* expression in normal cells. Thus, we predict that the two functions—antiapoptotic and telomere capping—are distinct and non-overlapping. In the future, it would be interesting to test whether distinct h*TR* structural domains are involved in these two functions as the anti-apoptotic function might be governed by h*TR*'s P1 helix, while the telomere capping function likely relies on KU interacting with its 3′ CR7 motif (nt 404–451). Testing the ability of a P1 helix mutant to protect cells against apoptosis may be indicative. On the other hand, overexpressing, in ALT$^+$ cells, an h*TR* mutant that lacks the 3′ CR7 motif, may help to further test whether this region is indeed involved in the regulation of telomeric RPA abundance.

NHP2 AND THE NON-CANONICAL FUNCTION OF h*TR* IN REGULATING RPA AT ALT$^+$ TELOMERES: AN INTRIGUING LINK

Our recent study showed that h*TR*'s function in promoting RPA eviction from ALT$^+$ telomeres does not involve the NHP2 chaperone[18]—otherwise required for its functional assembly into the telomerase complex [5],[34],[35]. The role of the other h*TR* chaperones, DKC1, NOP10, TCAB1, and GAR1, in regulating h*TR*'s ability to promote RPA eviction has not been tested. Considering the shared overlapping binding sequences/domains on h*TR* for KU70/80 and NHP2 (Figure 3), it thus seems plausible that the protein interaction partner of h*TR* could determine its engagement in either the canonical telomerase activity or the non-canonical telomeric RPA regulation. In that context, it was surprising to observe that NHP2 protein levels get naturally downregulated in h*TR*-expressing ALT$^+$ cell lines, whether in our laboratory-generated cellular models or in naturally occurring ALT$^+$ cancer cell lines [18]. When investigating the functional consequences of reduced NHP2 protein levels, we found that, while NHP2

downregulation does not rescue telomeric RPA abundance, it reduces the h*TR*-dependent increase in 53BP1 DNA damage marker recruitment at ALT telomeres, thus retaining telomeric DDR activation at low levels [18]. This regulation of 53BP1 foci by NHP2 is independent of h*TR* expression or telomerase activity and adds to the NHP2's growing repertoire of other additional important roles such as ribosomal RNA biogenesis and regulation [35]. Since KU and NHP2 share overlapping binding domains on h*TR*, it may be difficult to generate separation-of-function mutants of h*TR* to address the impact of interaction with one protein over the other. Similarly, as both NHP2 and KU have functions beyond their interaction with h*TR*, knocking down individual proteins may not give clear answers. Ideally, here too, one must map and identify the amino acid residues of NHP2 and KU that are key for binding to h*TR*. It is also tempting to speculate that, in normal somatic cells devoid of hTERT, NHP2 binding to h*TR* may be reduced and h*TR* may preferentially interact with KU, thus actively facilitating the telomerase-independent telomere capping activity. Biochemical binding assays using in vitro transcribed h*TR* and purified hTERT, KU and NHP2 proteins may help reveal if removing hTERT from the mix favors h*TR* binding to KU rather than NHP2.

CONCLUSIONS AND PROSPECTS

Although the *TR* component of telomerase has evolutionarily diverged in its sequence, length, and structure, these divergent structures share some specific features critical for *TR* to function as the TERT complement for telomerase function. The role of *TR* as telomerase RNA template is the best characterized one, with a lot of our understanding coming from studies of clinically observed h*TR* mutations in telomere biology disorders. Interestingly, all pathological h*TR* mutations known to date are of heterozygous nature, with no reported biallelic or null mutations, strongly indicating that, unlike m*TR*, h*TR* is an essential gene,[36] arguably through its anti-apoptotic and telomere capping functions. Along this line, while the majority of clinically observed h*TR* mutations sit around the pseudoknot domain, there are exceptions that do not affect its role as telomerase RNA template, including mutations in the 5′ end of h*TR*—a region not shared by m*TR*.[37,38,39]

The growing body of evidence for non-canonical roles of h*TR* adds to the emerging roles for lncRNAs as drivers and regulators of several key cellular pathways. If h*TR* functions have been mostly studied in the context of telomere length maintenance in cancers, it is imperative to understand the functions of h*TR* in normal somatic cells with naturally silenced h*TERT* gene. Our hypothesis that h*TR* serves as the regulator to switch the DNAPKcs-hnRNPA1 complex from an RPA-depositing to an RPA-eviction complex offers an elegant perspective on regulation of telomere capping in normal cells.

It remains unclear why so many distinct functions are performed by a single lncRNA molecule and how exactly the dynamics between these distinct functions is regulated based on h*TR*'s interaction partners—both RNA and proteins. Future experiments will likely reveal important aspects of the multiple roles of h*TR* that are of particular relevance in normal cells.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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