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The biogenesis and regulation of telomerase holoenzymes

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Abstract

Chromosome stability requires a dynamic balance of DNA loss and gain in each terminal tract of telomeric repeats. Repeat addition by a specialized reverse transcriptase, telomerase, has an important role in maintaining this equilibrium. Insights that have been gained into the cellular pathways for biogenesis and regulation of telomerase ribonucleoproteins raise new questions, particularly concerning the dynamic nature of this unique polymerase.

Linear chromosome ends, or telomeres, must be distinguished from sporadic DNA breaks so that only broken ends are rejoined by repair. Telomeres of eukaryotic nuclear chromosomes typically harbour an array of simple-sequence repeats. These telomeric repeats were first characterized in the macronucleus of the ciliate *Tetrahymena thermophila* as ~50 copies of 5'-T₂G₄-3' (REF. 1). In vertebrates, telomeres have ~1000 copies of 5'-T₂AG₃-3' (REFS 2,3). Telomeric repeat DNA assembles with numerous proteins to create an end-protective chromatin structure (reviewed in REFS 4–7).

Sequence erosion of terminal repeats is inherent in each round of genome replication. The replenishment of telomeric repeats can occur by recombination-based mechanisms, but it more commonly involves the extension of the 3' ends. This *de novo* extension reaction is mediated by the essential eukaryotic reverse transcriptase (RT), telomerase. The telomerase ribo-nucleoprotein (RNP) carries an internal template for telomeric repeat synthesis and specifically recognizes chromosome ends as substrates. Telomerase-mediated repeat synthesis can balance the loss of chromosome ends that occurs during genome replication and damage to maintain the homeostasis of telomere length. By contrast, if this balance is not maintained, cumulative loss or gain of telomere length eventually perturbs the insulating function of telomeric chromatin. Typically, compromised telomere structure halts cell proliferation as a response to DNA damage⁴. Otherwise, cell division without sufficient telomeric repeats induces large-scale genomic instability^{8,9}.

Competing interests statement

The author declares no competing financial interests.

DATABASES

The following terms in this article are linked online to:

Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>.

EST1 | *EST2* | *EST3* | *EST4*

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Cbf5 | dyskerin | GAR1 | TERT | NHP2 | NOP10

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The important role of telomerase activity in the maintenance of genome integrity suggests that telomerase should be a constitutive 'housekeeping' enzyme. Instead, it has been shown that telomerase is extensively regulated. Multicellular organisms have developmental, tissue-specific and stress-responsive strategies for telomerase repression^{10–12}. The inactivation of telomerase and telomere-length maintenance in human somatic cells has been proposed to function as a tumour-suppressor mechanism^{13–15}. Inactivation of telomerase might also be required for quiescence, differentiation and death of some cell types¹⁶. However, cumulative telomere erosion limits the renewal capacity of highly proliferative human cell lineages in the skin and blood^{17, 18}. Active telomerase has been detected in extracts of human cells from early embryogenesis, the germline, a subset of epithelial and lymphoid progenitors and almost all types of cancer^{12, 17}. Because cancer cells strongly upregulate telomerase activity and remain dependent on telomerase function for viability, telomerase inhibitors are under development as potential anti-cancer therapeutics^{8, 19}.

Telomerase studies address questions with broad relevance to diverse fields of current research. Recent molecular and biochemical studies of telomerase have focused on the properties of the enzyme as a specialized polymerase, a dynamic RNP complex and a factor that binds to specific regions of each chromosome. This review aims to draw together insights on telomerase in its physiological context, with emphasis on molecular and biochemical characterization across three model systems: budding yeast, vertebrates and ciliates. After introducing telomerase, successive sections describe the requirements for endogenous RNP biogenesis, the requirements for activity on telomeres, and last, other regulatory factors that are involved in telomerase activity. The review concludes with a discussion on enzyme structure and mechanisms of activity. It is important to refine and broaden the approaches that are used to study telomerase in the physiological context due to direct implications of telomerase deregulation and deficiency in human disease^{18, 20, 21}.

An RNA template for DNA synthesis

RTs are best known as viral proteins that copy an RNA genome into DNA. The discovery of a eukaryotic RT occurred in the pursuit of an understanding of telomere dynamics. The first studies of telomere structure were performed in ciliated protozoa, which share a developmentally regulated process of telomere synthesis at formerly non-telomeric sites of chromosome fragmentation²². A similar process was experimentally induced in *Saccharomyces cerevisiae* by introducing plasmids with ciliate-chromosome termini that gained extra caps of the distinct yeast telomeric repeat²³. Cell extracts from the ciliate *T. thermophila* yielded biochemical evidence of an enzyme that could extend a single-stranded primer with single-stranded telomeric repeats²⁴. Initially, the persistence of this activity in micrococcal nuclease-treated extracts led to this enzyme's designation as telomere terminal transferase²⁴, with potential analogy to the sequence-specific nucleotide-transferase CCA-adding enzyme, which completes or restores the 3' end of mature transfer RNAs²⁵. Subsequently, the repeat-synthesis activity was recognized to be nuclease-sensitive in a unique way. The enzyme, now termed telomerase, required a specific RNA molecule with an internal sequence that could be used as a template for the synthesis of telomeric repeats^{26, 27}. RNase-sensitive synthesis of telomeric repeats was next shown in cell extracts from the divergent spirotrichous ciliates *Oxytricha nova* and *Euplotes crassus*^{28, 29}, and then leapt onto the main stage following its detection in extracts from cultured human cancer cells³⁰.

Pioneering molecular genetics studies in *T. thermophila* established that the integral telomerase RNA subunit (TER) provides the template for repeat synthesis at chromosome ends^{31, 32}. Curiously, there are exceptions to this rule. Repeat synthesis in some organisms, such as the ciliate *Paramecium tetraurelia*, is not strictly determined by the template³³.

Also, repeat synthesis can variably fail to extend for the entire length of the template, as occurs in *S. cerevisiae*^{34,35}. Our current knowledge of the primary sequence of TER is restricted to yeasts, ciliates, vertebrates and one virus^{36,37}.

The catalytic activity of telomerase requires TER and the protein telomerase RT (TERT). TERT harbours a central region that has homology with the active-site motifs of the viral RT enzymes^{38–40}. Although recombinant TERT cannot extend a typical RT substrate of annealed template and primer⁴¹, it can catalyse a weak nucleotide-transfer reaction⁴². Many organisms encode a single isoform of TERT. However, *E. crassus* has three genes that encode distinct TERT proteins and show differential regulation that is correlated with changes in telomerase activity⁴³. Also, human TERT pre-mRNA is alternatively spliced in a manner that is predicted to alter telomerase function at telomeres⁴⁴. The biological significance of these examples of isoform diversity remains to be tested experimentally.

In telomerase-sized RNP enzymes, such as eukaryotic RNase P, multiple proteins assemble with each other and their RNA in a tightly coupled manner⁴⁵. The depletion of any subunit of the endogenously assembled RNase P holoenzyme affects RNP accumulation and/or its activity in cell extracts. By contrast, evidence from studies in yeasts, vertebrates and ciliates strongly indicates that endogenous assembly of telomerase holoenzymes is more complicated. The physiological pathways of telomerase biogenesis and regulation generate a diversity of complexes that contain TER and/or TERT. Some activities of TER and TERT have been proposed that are independent of telomere synthesis, and in some cases independent of each other^{16,46–49}. In this article, the term ‘telomerase RNP’ will be used to describe all endogenously assembled complexes that contain TER. The term ‘telomerase holoenzyme’ will be used to describe the subset of these RNPs that also contain TERT. However, this working definition of telomerase holoenzyme might need to be reconsidered as we gain new insights into the dynamics of telomerase complexes *in vivo*.

Following the directions for RNP assembly

Purified TER and TERT do not form active RNP. However, TER and TERT can be combined in the presence of a crude eukaryotic cell extract such as rabbit reticulocyte lysate to generate catalytically active RNP⁵⁰. Similarly to many other RNP enzymes, telomerase relies on a step-wise cellular RNP biogenesis pathway to provide assembly specificity. As cells differ in their levels of telomerase RNP depending on growth conditions and cell type, this non-spontaneous process of endogenous telomerase RNP biogenesis also provides an opportunity for enzyme regulation.

Tightly associated TER-binding proteins that are required for the biological stability of telomerase RNP have been identified. These proteins are mandatory subunits of an endogenously assembled telomerase RNP, and they constitute the structural core of any telomerase holoenzyme. Dissociation of these proteins from TER is expected to induce RNP turnover. Notably, TERT is not required for the biological stability of yeast and vertebrate telomerase RNPs. In yeast, telomerase RNPs accumulate *in vivo* in the presence or absence of co-expressed TERT⁵¹. Similarly, human telomerase RNPs accumulate ubiquitously in cells regardless of the presence of telomerase activity in cell extracts⁵².

Telomerase RNP biogenesis in yeast

Several of the cellular requirements for generating a biologically stable telomerase RNP in budding yeast are similar to the requirements for the assembly of spliceosomal small nuclear (sn) RNPs^{53,54}. The yeast TER primary transcript is synthesized by RNA polymerase II, polyadenylated, capped on its 5' end and processed to remove the polyadenosine tail^{34,53} (FIG. 1a). Sequence inspection suggested the presence of a uridine-rich consensus motif that

is required for association with Sm proteins⁵⁴. Sm proteins were originally identified as human autoantigens and were later shown to co-assemble into a hetero-heptameric complex that provides the biological stability of many snRNAs⁵⁵. Mutation of the consensus Sm-binding site in *S. cerevisiae* TER or the genetic depletion of the Sm D1 protein drastically reduces telomerase RNP accumulation⁵⁴. Importantly, epitope-tagged Sm D1 co-purifies the active telomerase in cell extracts, which is confirmation of an association with telomerase holoenzyme. Additional aspects of the yeast telomerase RNP biogenesis pathway remain to be investigated, including whether the shuttling of telomerase RNP between the nucleus and the cytoplasm^{56,57} is important for the specificity of the assembly pathway.

H/ACA-motif RNP assembly in vertebrates

Studies of human TER led to the first insights about the endogenous pathways of telomerase RNP biogenesis and brought attention to the role of telomerase deficiency in human disease (BOX 1). The human TER primary transcript is synthesized by RNA polymerase II, capped on its 5' end, internally modified, and processed at its 3' end to generate the mature, functional TER^{17,52,58} (FIG. 1b). The proper processing and stability of human TER are dependent on a motif that is formed by the 3' half of the molecule⁵⁹. This H/ACA motif is common to vertebrate TER and conserved RNA families that guide the post-transcriptional modification of non-protein-coding RNAs⁶⁰. *In vivo* studies of general H/ACA-motif RNP assembly have shown the co-transcriptional binding of three proteins essential for RNP accumulation — dyskerin (Cbf5 in yeast), NHP2 and NOP10 — and the subsequent joining of a fourth protein, GAR1 (REF. 60). Human TER also assembles with these four H/ACA-motif RNP proteins. Importantly, endogenous and epitope-tagged versions of these proteins co-purify active telomerase from cell extract, as expected for an association with telomerase holoenzyme^{61–64}.

Box 1/Telomerase deficiency in human disease

Telomerase deficiency in humans was first described in the disease dyskeratosis congenita (DC). Patients with DC share signs of insufficient cellular renewal in the skin and blood, including nail loss and low blood-cell counts, and they die primarily from bone-marrow failure^{20,21}. The predominant X-linked inheritance of DC arises from substitutions in the RNA-binding protein dyskerin. Dyskerin, NHP2, NOP10 and GAR1 (which are all involved in RNP biogenesis) assemble together on H/ACA-motif RNAs to form ribonucleoproteins (RNPs) that modify ribosomal RNAs and other functional RNAs by conversion of uridine to pseudouridine⁶⁰. The H/ACA-motif RNAs determine the sites of modification by hybridization to the target sequences. Dyskerin, NHP2 and NOP10 are essential for the cellular accumulation of the H/ACA-motif RNAs. Also, dyskerin contains the active site for the modification reaction. Cells from patients with X-linked DC that express variants of dyskerin have normal levels of H/ACA-motif RNAs and pseudouridine modifications, but accumulation of the telomerase RNA subunit (TER) is reduced^{61,152}. Telomerase deficiency leads to premature telomere shortening, which in turn limits the renewal capacity of highly proliferative cell types in skin and blood.

The less common, and generally less severe, autosomal dominant inheritance of DC was next investigated, and the disease was shown to coincide with the heterozygous expression of a TER variant¹⁵³. Shortly thereafter, patients with aplastic anaemia were also shown to have heterozygous expression of a TER variant¹⁵⁴. Mutations in the gene that encodes telomerase reverse transcriptase (TERT) have recently been detected in patients with blood diseases¹⁵⁵. These, and potentially other, distinct human disorders of cell proliferation can all be derived from telomerase deficiency, yet they differ in clinical

presentation because the extent and the mechanism of telomerase inhibition affects the spectrum of tissue involvement^{17,18,20,21}.

Although the consensus H/ACA motif is necessary and sufficient to direct RNP assembly for most H/ACA-motif RNAs, it is not sufficient for the assembly of a telomerase RNP that retains full-length TER^{59,65}. The extra requirement for TER accumulation involves the loop of the H/ACA-motif 3' hairpin, which is proposed to interact with an unknown RNP biogenesis factor^{64,65}. This 3' loop has two separable roles; it is involved in RNP biogenesis and in RNP enrichment in subnuclear compartments that are known as Cajal bodies^{58,66}. The observed enrichment of telomerase RNP in Cajal bodies is not required for RNP biogenesis or catalytic activation^{58,66}, and so the biological significance of this localization remains to be determined. It will be important to gain insight into the missing details of the vertebrate telomerase RNP biogenesis pathway for two reasons. If additional TER-specific protein–RNA interactions are discovered, they can be used to develop screens for clinically useful telomerase inhibitors. Also, knowledge of the vertebrate-specific features of telomerase RNP biogenesis might provide clues leading to the discovery of vertebrate-specific strategies for the regulation of enzyme functions *in vivo*.

Insights into enzyme assembly in ciliates

Ciliates have a unique pathway for telomerase RNP biogenesis. The ciliate TER primary transcript is synthesized by RNA polymerase III and not processed, leaving a 3' tail of polyuridine that was added during transcription termination²⁷ (FIG. 1c). Oligonucleotide-based telomerase affinity purification from *Euplotes aediculatus* recovered TERT and a 43 kDa protein (p43) with a divergent La motif for RNA-binding^{67,68}. On the other side of the ciliate family tree, *T. thermophila* telomerase affinity purification using endogenously expressed epitope-tagged TERT identified several holoenzyme proteins, including one of 65 kDa (p65) with the same divergent La motif⁶⁹. Importantly, antibodies against p43 or p65 deplete telomerase activity from cell extract, and epitope-tagged p65 co-purifies telomerase activity, which demonstrates the association of these proteins with telomerase holoenzymes^{68,69}. Recombinant p43 or p65 binds directly to TER, with a requirement for the base-paired stems of TER rather than the single-stranded regions that are a typical requirement for La-motif-protein binding^{70–72}. Genetic depletion of *T. thermophila* p65 drastically reduces TER and TERT accumulation without affecting other RNPs⁶⁹, which indicates that this protein has an essential role in the cellular biogenesis of stable telomerase RNP.

Results from recent studies with *T. thermophila* p65, TER and TERT offer a rationale for the evolution of multi-step telomerase RNP biogenesis pathways. In an entirely recombinant system, the p65–TER interaction greatly stimulates the TERT–TER interaction through a conformational change in the bridging TER^{71,72}. This finding indicates that a tightly associated TER-binding protein functions both to stabilize telomerase RNP and to fold TER into a structure that is favourable for TERT binding and enzyme function. In the future, it will be important to use direct structural assays to determine p65-mediated changes in TER conformation and to test the generality of these findings for telomerase RNP biogenesis in other organisms.

An on/off relationship with chromosome ends

Mechanisms that govern telomerase interaction with telomeres have been studied mostly from the telomere side⁷³. To accomplish end protection, a telomere should deny enzymes access to the 3'-OH end of the chromosome (FIG. 2, end-protected telomere). Replication and processing reactions are proposed to open the telomere to potential remodelling (FIG. 2, open telomere). The open telomere can then attract enzymes or binding proteins that

generate a structure that allows elongation by telomerase (FIG. 2, telomerase-enabled telomere). Within a cell, each telomere must function in an autonomous fashion to solicit more or less lengthening, depending on the differential of its own length from the homeostasis.

It is less obvious why the engagement of telomerase with the telomere is regulated from the telomerase side. This level of regulation has been particularly well established in the physiological context of blood cells, in which some 'telomerase-positive' cell populations still suffer telomere erosion^{17,74}. Perhaps telomerase-subunits need to be released from sites of safe storage (FIG. 2, inhibitory sequestration). Or, a change in RNP conformation or composition could be required to gain full DNA-binding specificity (FIG. 2, telomerase-enabled holoenzyme). Alternatively, factors that are required to dissociate telomerase from the telomere could be over-active (FIG. 2, right half). In addition to these protein-protein and protein-DNA interactions, telomerase interaction with the telomere might also be regulated by DNA-DNA interactions (BOX 2).

Recruitment to the telomere in yeast

The first genes that were shown to have roles in telomere-length maintenance were identified in *S. cerevisiae*. Mutant strains with defects in telomere replication were isolated by screening for several phenotypes, including progressive telomere erosion, which led to the identification of the ever shortening telomere (*EST*) genes^{75,76}. *EST2* encodes TERT³⁸ and *EST4* encodes a partial loss-of-function allele of the telomere-binding protein Cdc13 (REFS 76,77). The *EST1* and *EST3* genes encode proteins that function only, at least by genetic criteria, in telomerase-dependent telomere-length maintenance. Strains that lack Est1 or Est3 show proliferation-dependent telomere erosion, yet they retain telomerase activity in cell extracts⁵¹. Importantly, epitope-tagged Est1 and Est3 co-purify active telomerase from cell extract, as is expected for holoenzyme proteins⁷⁸. Est1 associates with TER independently of TERT, probably as a direct interaction (FIG. 1a), whereas Est3 associates with TER only through TERT⁷⁸⁻⁸⁰.

Results from directed interaction assays and chromatin immunoprecipitation experiments indicated that there are at least two mechanisms for the physical recruitment of telomerase to the telomere^{77,81}. The first interaction, which is correlated with telomere elongation, occurs in the S phase of the cell cycle and involves Cdc13 (REF. 82). A second interaction, which is evident in the G1 phase, is mediated through the association of TER with Ku, a DNA-end-binding protein⁸¹. Ku is not essential for telomere maintenance, but the disruption of the direct Ku-TER interaction reduces telomere length and reduces chromosome healing at DNA breaks⁸³. The factors that discriminate these two modes of telomere association are not obvious, as Ku binds to TER at a site that does not overlap with other known protein-TER interactions^{80,84} (FIG. 1a). Est1 levels rise on S-phase entry and drop with S-phase exit⁸², so it might be that either an excess of Est1 triggers the holoenzyme's S-phase mode of engagement or that the removal of Est1 from the holoenzyme forces it out of the S-phase mode.

Recruitment to vertebrate and ciliate telomeres

The mammalian heterogeneous nuclear (hn) RNP proteins A1 and C have been proposed to link the telomerase holoenzyme to telomeres, potentially by bridging TER and single-stranded DNA^{85,86}. These and other abundant hnRNP proteins package a broad spectrum of RNA polymerase II transcripts, so it is therefore difficult to establish a function for hnRNPs that is specific to the context of the telomerase holoenzyme. Recent studies indicated that hnRNP A1 stimulates telomerase activity by counteracting single-stranded product DNA folding⁸⁷. Other factors that might link the telomerase holoenzyme to

telomeres include three vertebrate proteins that have been characterized based on limited homology with the budding and fission yeast Est1 (REF. 88). Two of these factors have been described as holoenzyme proteins^{89,90}, but as these proteins have important functions beyond the regulation of telomerase activity, their roles specific to the context of the holoenzyme might be difficult to establish.

In *T. thermophila*, subunits of the telomerase holoenzyme affect the ability of the enzyme to extend chromosome substrates (FIG. 1c). The genetic depletion of the p45 telomerase protein induces telomere shortening without affecting RNP accumulation *in vivo*⁶⁹, which is similar to the null phenotype of *EST1* and *EST3* knockouts in budding yeast. Importantly, antibodies against *T. thermophila* p45 deplete telomerase activity from cell extract, and epitope-tagged p45 co-purifies active telomerase, which confirms the association of p45 with the telomerase holoenzyme⁶⁹. It will be interesting to determine and compare the mechanisms by which the *S. cerevisiae* and the *T. thermophila* holoenzyme proteins activate telomerase for function at telomeres, because these two single-celled eukaryotes have quite distinct telomere and telomerase structures.

Box 2|Folding of guanosine-rich single-stranded telomeric repeat DNA

Single-stranded telomeric repeat DNA that contains tracts of G nucleotides can fold to form stable intramolecular or intermolecular G-quartet structures. G nucleotides in a planar configuration form Hoogsteen base-pairs, each constituted from one canonical and one non-canonical pairing edge of the base. Four telomeric repeats donate G-tracts such that consecutive Gs within a repeat are stacked and pair in successive quartets. G quartets generally reduce the efficiency of primer extension by telomerase *in vitro*^{156, 157}.

Does this type of DNA folding regulate the interaction of telomerase with endogenous telomere substrates? In spirotrichous ciliates, telomere-binding proteins have been proposed to promote G-quartet formation¹⁵⁸. In other organisms, G-rich-strand folding is thought to be actively resolved to prevent accelerated telomere shortening¹⁵⁹. It remains to be established whether G quartets or other structures that are formed by G-rich single-stranded telomeric repeat DNA have a direct role in modulating telomerase activity at telomeres.

Dynamic and transient interaction partners

The holoenzyme proteins that are required for telomerase RNP accumulation, activity and function at the telomere make essential contributions to telomere maintenance. Although the characterization of telomerase-associated proteins is far from complete, numerous additional factors have been suggested to interact with the telomerase holoenzyme and/or intermediates in its biogenesis and regulation. Among their many roles, these factors probably determine the dynamic distributions of TER and TERT within a cell. Particularly in vertebrate cells, TER and TERT localizations have been described to vary across the cell cycle without mutual coordination^{66, 91, 92}. This dynamic regulation could be partially directed by cellular chaperones that are required for holoenzyme assembly, disassembly and degradation. The physiological significance of the dynamics of telomerase subunits will be important to investigate in future studies.

Dynamic traffic

Human TER accumulates in Cajal bodies^{66, 91–93}, which are readily detectable in a subset of cell types⁹⁴. TER also associates with nucleoli^{59, 92} and colocalizes with a subset of telomeres in S phase^{91, 92}. Recombinant human TERT can be detected in the nucleoplasm, nucleoli and Cajal bodies^{93, 95–99}, whereas endogenous TERT has been localized to the

nucleoplasm, nucleoli, nucleoplasmic foci and telomeres^{92,99-101}. TERT localization can change in response to DNA damage or the expression of an oncogene⁹⁸. TERT also seems to be redistributed from the nucleus to the cytosol or vice-versa; this translocation correlates with telomerase activation, cellular stress and/or protein phosphorylation¹⁰²⁻¹⁰⁵.

TER and the p43 holoenzyme protein from *O. nova* or *E. aediculatus* accumulate in dispersed macronuclear foci that are separated from chromatin, and only a small percentage of this pool is transiently recruited to zones of DNA replication^{70,106,107}. *Euplotes crassus* TERT localizes transiently to replication bands without obvious concentration in foci¹⁰⁸, whereas *E. aediculatus* TERT shows more dispersed macronuclear localization¹⁰⁷. In yeast, the endogenous levels of TER and TERT have not been detected using cytological methods. Overexpression studies have shown that TER accumulates in the nucleoplasm, whereas TERT accumulates in the nucleolus^{56,57}. However, their co-overexpression shifts the localization of TERT into the nucleoplasm with TER. In yeast, the nucleolar localization of TERT that is not associated with TER is consistent with competition for TERT binding between TER and the nucleolar ribosome-biogenesis protein PinX1 (REF. 109). In comparison, human PINX1 has been proposed to bind to and inhibit the activity of the telomerase holoenzyme^{110,111}.

Chaperones of assembly and destruction

Transient associations of TER and TERT with chaperone activities occur during the assembly, disassembly and degradation of telomerase complexes. Based on well-established precedents for the assembly of Sm proteins and H/ACA-motif RNA-binding proteins^{55,60}, the pathways for telomerase RNP biogenesis in budding yeast and vertebrate cells must involve the participation of RNP-assembly chaperones. The heat-shock protein-90 (HSP90) and p23 chaperones associate with human TERT, and the HSP90 inhibitor geldanamycin reduces recombinant or endogenous telomerase activity that is assayed *in vitro*^{112,113}. Geldanamycin also induces TERT ubiquitylation and degradation by the proteasome, which is mediated by the E3 ubiquitin ligase MRKN1 (REF. 114).

With chaperones, it can be difficult to discriminate whether an association reflects an involvement in the normal assembly or disassembly pathway of a complex, or an involvement in the recycling or disposal of a misfolded, off-pathway complex. The *T. thermophila* p80 and p95 proteins and vertebrate TEP1, which were originally characterized in association with TER and telomerase activity, are much more abundant than telomerase and are genetically dispensable for telomerase function *in vitro* and *in vivo*^{69,115-117}. Based on the bioinformatic prediction of p80 homology with Ro¹¹⁸, a protein that is thought to detect misfolded RNA or RNP *in vivo*¹¹⁹, p80 could have a general function in RNP recycling or degradation. Telomerase could be a physiological target of regulation by RNP recycling or degradation, which potentially accounts for telomere lengthening in *T. thermophila* strains that lack p80 and/or p95 (REF. 115). However, that p80 and/or p95 interact with TER under disruptive purification conditions is more consistent with RNP rearrangement in cell extract⁶⁹. Likewise, cell-extract-induced protein–RNA interactions could account for the associations of human TER and telomerase holoenzyme with abundant RNA-binding proteins such as La, Staufen, and the ribosomal protein L22 (REFS 120,121). *In vivo* crosslinking assays to test RNP composition prior to cell lysis¹²² have been developed for studies of more abundant RNPs. It will be important to apply these assays to studies of telomerase as they could provide insights into the physiological interaction partners of telomerase holoenzymes.

The telomerase catalytic cycle

Compared to nucleic-acid synthesis that is performed by other polymerases, telomerase-mediated telomeric repeat synthesis requires a more elaborate coordination of template, substrate and active site. The internal template must be brought to the active site in a manner that excludes the regions beyond the 5' and 3' template boundaries. The substrate must engage sequence-specific single-stranded-DNA-binding sites to give only its extreme 3' end the potential to pair with the template. With each successive nucleotide addition, architectural changes of the template and product in relation to the active site must occur (FIG. 3; see legend for details). To keep these moving parts in coordination, strong interactions that remain fixed relative to the active site should anchor the template and substrate in the RNP complex (FIG. 3; BOX 1).

Telomerase holoenzymes from different organisms, or from different developmental stages of the same organism, generate surprisingly diverse profiles of product synthesis^{123, 124}. Little is known about which features of telomerase activity *in vitro* affect its function at telomeres *in vivo*. Is it significant that relatively long products are synthesized by human and *T. thermophila* holoenzymes, whereas short products are synthesized by the mouse and yeast holoenzymes? Is the structure of the chromosome end influenced by the phylogenetic variation in the repeat permutation that is generated by synthesis to the template 5' end? To address these questions, more knowledge is required about the structures and mechanisms that direct repeat synthesis. To date, high-resolution structures have been determined only for isolated motifs of TER and for an N-terminal domain of *T. thermophila* TERT^{125, 126}.

Coordinated sites of interactions

The internal template is defined not by its own sequence but instead by flanking regions that establish the 5'- and 3'-template boundaries. The *T. thermophila* TER 5'-template boundary is determined, at least in part, by tight binding of a template-flanking motif to TERT¹²⁷. In other organisms, the region of TER that is adjacent to the 5' end of the template forms a base-paired duplex that is required for maintaining the 5' template boundary^{128, 129}. These TER-TERT and TER-TER interactions could occur in combination to reinforce the necessary halt of DNA synthesis at the 5' end of the template. How the 3' end of the template is defined is less clear. Elongation of an entirely non-telomeric-sequence primer by ciliate telomerases is mediated through the addition of a precise permutation of the telomeric repeat, which indicates that the template has a default position in the active site. The 3'-flanking region of the *T. thermophila* TER template can bring an exchangeable oligonucleotide template to the active site¹³⁰. It is therefore likely that at least transient TER-TERT or TER-TER interactions 3' of the template help to position the template 3' end. To correctly extend 3' substrate ends paired in variable register with the template, the template must be able to move variable distances from the default position by forming a template-primer duplex.

The mechanisms that underlie the recognition of a single-stranded-DNA substrate seem surprisingly variable between holoenzymes and have not yet been well characterized^{123, 124}. Activity assays, interaction assays and high-resolution structure have defined a binding surface for single-stranded DNA that is adjacent to the template hybrid, termed PAS1 (primer/product alignment/anchor site-1). DNA interactions with PAS1 (FIG. 3) are proposed to contribute to primer-binding affinity, alignment of the substrate 3' end and a limited extent of repeat-addition processivity^{125, 131-133}. The extensive characterization of the primer preferences of ciliate and vertebrate telomerase holoenzymes indicates that these telomerase enzymes have additional DNA-interaction specificity (PAS2; FIG. 4b). PAS2 sites could be contiguous with or separated from PAS1 and are proposed to account for the enhanced binding affinity of longer primers, high repeat-addition processivity and the

efficient elongation of primers with a 5'-telomeric sequence seed and non-telomeric 3' end^{123, 124}.

Functional domains of TERT

TERT domains (FIG. 4a) have a mostly unknown architecture, so only a speculative working model for their coordination can be proposed (FIG. 4b). The TERT high-affinity RNA-binding domain (TRBD) is sufficient for interaction with *T. thermophila* TER *in vitro* or human TER *in vivo*¹³⁴. Bacterially expressed *T. thermophila* TRBD does not require chaperones for assembly with TER, which indicates that the chaperone requirement of full-length TERT might reflect a need to expose, rather than fold, the TRBD¹³⁵. Additional sites of TERT-TER contact have been proposed for *T. thermophila* and human TERTs. However, these sites have relatively low binding affinity and it is therefore difficult to study the specificities of their interactions.

The TERT essential N-terminal (TEN) domain is highly conserved among vertebrate proteins, which is an exception to the generally low level of sequence conservation that occurs beyond the active site. Activity assays and high-resolution structure analysis implicate the *T. thermophila* TEN domain as a PAS1 contact (FIG. 4b). The TEN domains of yeast and human TERTs could provide PAS1 contacts as well^{125, 136–138}. It is tempting to speculate that the *T. thermophila* TEN domain associates with the 3'-flanking region of the template (FIG. 4b) for at least part of the catalytic cycle^{135, 139}. The TERT C-terminal extension (TEC) is not essential for yeast holoenzyme function *in vivo*¹⁴⁰. However, substitutions of the human TERT C terminus abrogate its physiological function in the same manner as some substitutions in its TEN domain^{141, 142}. One plausible model to account for these observations is that an interaction between TEC and TEN could provide PAS2 (FIG. 4b).

Tetrahymena thermophila telomerase RNPs assembled *in vivo* or *in vitro* are purified in complexes that contain a single TERT and a single TER^{69, 143, 144}. The *S. cerevisiae* telomerase holoenzyme also purifies from extract without evidence for subunit multimerization, unless single-stranded DNA has been added to the extract^{145, 146}. Human telomerase RNPs reconstituted *in vitro* are purified as interdependent multimers of TER and TERT, with functional interference from the co-assembly of mutant and wild-type TER templates¹⁴⁷. Human telomerase RNPs that were reconstituted *in vivo* under conditions of wild-type and mutant TER co-expression did not show this type of functional interference^{65, 148–150}. Notably, the TEN domain of one TERT polypeptide and the TRBD, RT, and TEC domains of another can complement each other in function to generate enzyme activity *in vitro*¹⁵¹. Perhaps the human TERT TEN and TEC domains form an intramolecular interaction during their assembly with TER *in vivo*, whereas *in vitro* they assemble preferentially by intermolecular complementation. Human TERT multimerization has been proposed to account for the large mass of the endogenous holoenzyme, which even after some disassembly exceeds the expected mass for a single TERT and TER alone¹⁴⁷. This additional mass can instead be constituted by the holoenzyme proteins that direct telomerase accumulation and function in an endogenous context.

Conclusions

It is now becoming clear that there are intricate pathways of telomerase assembly and regulation. First, the biogenesis of the telomerase RNP proceeds through sequential steps of protein-RNA interaction. Budding yeast, vertebrates and ciliates use different TER-binding proteins to produce a biologically stable telomerase RNP. The assembly of RNP with TERT then leads to RNP activation, which seems to be a dynamic process. Additional holoenzyme proteins give the enzyme an ability to function on chromosome substrates. Last, TERT, TER

and single-stranded DNA form a network of protein–nucleic-acid interactions that orchestrate the proper positioning of the template and primer in the active site.

Knowledge that has been gained over the past decade has highlighted the questions that remain to be answered. The unexplained difference in results from reconstitutions of TERT and TER *in vitro* and *in vivo* indicates a gap in our understanding of their interaction specificity. How does endogenous telomerase biogenesis bring the inactive RNP and TERT together? Is this assembly step reversible and is disassembly used to regulate the level of enzyme activation? Another important direction of future progress will be to understand the mechanisms that provide specificity for telomerase elongation of primer substrates *in vitro* and chromosome ends *in vivo*. How do holoenzyme proteins stimulate activity on telomere substrates? Do they induce a conformational change that locks the enzyme onto DNA or do they bind to DNA directly? Insights into the cellular requirements for telomerase biogenesis and regulation will hopefully lead to improved telomerase reconstitution *in vitro*. Also, studies of recombinant telomerase will provide more insights into the workings of an enzyme that is uniquely devoted to repeat synthesis.

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Glossary

Telomere	The terminal domain of an intact chromosome that protects DNA termini from inappropriate degradation, recombination or fusion with other chromosome ends.
Ciliates	Large single-celled eukaryotes that differentiate both the germline and the somatic versions of their nucleus within a common cytosol.
CCA-adding enzyme	The enzyme that adds or repairs the CCA-3' end that is shared by all functional transfer RNAs and that catalyses distinct, successive, sequence-specific nucleotide-transfer reactions.
RNase P	An RNP with an ancestral catalytic RNA that performs a phosphodiester cleavage reaction to generate the mature 5' end of transfer RNAs.
Small nuclear (sn) RNP	Non-coding snRNA assembles as an sn ribonucleoprotein (snRNP), which typically functions in the nucleus in RNA processing.
H/ACA motif	An RNA motif with a consensus structure that includes, from 5' to 3' with conserved spacing, a hairpin stem, a hinge sequence, a second hairpin stem and an ACA sequence.
Cajal body	A nuclear compartment that forms in a subset of cell types and contains factors that process, modify and assemble RNPs.
Ku	A heterodimeric protein that forms a basket-like structure for encircling DNA ends and that recruits the DNA-repair machinery to DNA breaks.

Heterogeneous nuclear (hn) RNP proteins	Abundant proteins that were initially identified by their association with bulk nuclear pre-mRNA and are now recognized to have additional more specialized functions.
Nucleolus	A nuclear compartment that forms by coupled transcription, processing and RNP assembly of ribosomal RNAs. It also has roles in sequestration of cell-cycle regulators, transcription factors and other molecules from their sites of action.

References

1. Blackburn EH, Gall JG. A tandemly repeated sequence at the termini of the extrachromosomal ribosomal RNA genes in *Tetrahymena*. *J. Mol. Biol* 1978;120:33–53. [PubMed: 642006]
2. Moyzis RK, et al. A highly conserved repetitive DNA sequence, (TTAGGG)_n, present at the telomeres of human chromosomes. *Proc. Natl Acad. Sci. USA* 1988;85:6622–6626. [PubMed: 3413114]
3. Meyne J, Ratliff RL, Moyzis RK. Conservation of the human telomere sequence (TTAGGG)_n among vertebrates. *Proc. Natl Acad. Sci. USA* 1989;86:7049–7053. [PubMed: 2780561]
4. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005;19:2100–2110. [PubMed: 16166375]
5. Bhattacharyya MK, Lustig AJ. Telomere dynamics in genome stability. *Trends Biochem. Sci* 2006;31:114–122. [PubMed: 16406636]
6. de Lange T. T-loops and the origin of telomeres. *Nature Rev. Mol. Cell Biol* 2004;5:323–329. [PubMed: 15071557]
7. de Lange, T.; Lundblad, V.; Blackburn, EH., editors. *Telomeres*. 2nd edn. Cold Spring Harbor Laboratory Press; 2006.
8. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. *Nature Rev. Genet* 2005;6:611–622. [PubMed: 16136653]
9. Maser RS, DePinho RA. Connecting chromosomes, crisis and cancer. *Science* 2002;297:565–569. [PubMed: 12142527]
10. Schatzlein S, Rudolph KL. Telomere length regulation during cloning, embryogenesis and ageing. *Reprod. Fertil. Dev* 2005;17:85–96. [PubMed: 15745634]
11. Djojsubroto MW, Choi YS, Lee HW, Rudolph KL. Telomeres and telomerase in aging, regeneration and cancer. *Mol. Cells* 2003;15:164–175. [PubMed: 12803478]
12. Forsyth NR, Wright WE, Shay JW. Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation* 2002;69:188–197. [PubMed: 11841477]
13. Harley CB. Telomere loss: mitotic clock or genetic time bomb? *Mutation Res* 1991;256:271–282. [PubMed: 1722017]
14. Sharpless NE, DePinho RA. Telomeres, stem cells, senescence, and cancer. *J. Clin. Invest* 2004;113:160–168. [PubMed: 14722605]
15. Shay JW, Wright WE. Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis* 2005;26:867–874. [PubMed: 15471900]
16. Blackburn EH. Cell biology: shaggy mouse tales. *Nature* 2005;436:922–923. [PubMed: 16107824]
17. Collins K, Mitchell JR. Telomerase in the human organism. *Oncogene* 2002;21:564–579. [PubMed: 11850781]
18. Wong JMY, Collins K. Telomere maintenance and disease. *Lancet* 2003;362:983–988. [PubMed: 14511933]
19. Shay JW, Wright WE. Telomerase: a target for cancer therapeutics. *Cancer Cell* 2002;2:257–265. [PubMed: 12398889]
20. Walne AJ, Marrone A, Dokal I. Dyskeratosis congenita: a disorder of defective telomere maintenance? *Int. J. Hematol* 2005;82:184–189. [PubMed: 16207588]

21. Mason PJ, Wilson DB, Bessler M. Dyskeratosis congenita — a disease of dysfunctional telomere maintenance. *Curr. Mol. Med* 2005;5:159–170. [PubMed: 15974869]
22. Yao MC, Chao JL. RNA-guided DNA deletion in *Tetrahymena*: an RNAi-based mechanism for programmed genome rearrangements. *Annu. Rev. Genet* 2005;39:537–559. [PubMed: 16285871]
23. Shampay J, Szostak JW, Blackburn EH. DNA sequences of telomeres maintained in yeast. *Nature* 1984;310:154–157. [PubMed: 6330571]
24. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* 1985;43:405–413. [PubMed: 3907856] The first description of telomere terminal-transferase activity.
25. Weiner AM. tRNA maturation: RNA polymerization without a nucleic acid template. *Curr. Biol* 2004;14:R883–R885. [PubMed: 15498478]
26. Greider CW, Blackburn EH. The telomere terminal transferase of *Tetrahymena* is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* 1987;51:887–898. [PubMed: 3319189]
27. Greider CW, Blackburn EH. A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* 1989;337:331–337. [PubMed: 2463488] The identification of telomerase RNA.
28. Zahler AM, Prescott DM. Telomere terminal transferase activity in the hypotrichous ciliate *Oxytricha nova* and a model for replication of the ends of linear DNA molecules. *Nucl. Acids Res* 1988;16:6953–6972. [PubMed: 3136437]
29. Shippen-Lentz D, Blackburn EH. Telomere terminal transferase activity from *Euplotes crassus* adds large numbers of TTTTGGGG repeats onto telomeric primers. *Mol. Cell. Biol* 1989;9:2761–2764. [PubMed: 2474761]
30. Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell* 1989;59:521–529. [PubMed: 2805070] Shows human telomerase activity *in vitro* and that it has many similarities with ciliate telomerase activity.
31. Yu G, Bradley JD, Attardi LD, Blackburn EH. *In vivo* alteration of telomere sequences and senescence caused by mutated *Tetrahymena* telomerase RNAs. *Nature* 1990;344:126–132. [PubMed: 1689810] Establishes that the internal template of telomerase RNA is copied into telomeric repeat DNA, and provides a new method for altering the telomeric repeat sequence for studies of telomere function.
32. Yu G, Blackburn EH. Developmentally programmed healing of chromosomes by telomerase in *Tetrahymena*. *Cell* 1991;67:823–832. [PubMed: 1934071]
33. McCormick-Graham M, Haynes WJ, Romero DP. Variable telomeric repeat synthesis in *Paramecium tetraurelia* is consistent with misincorporation by telomerase. *EMBO J* 1997;16:3233–3242. [PubMed: 9214639]
34. Singer MS, Gottschling DE. TLC1: template RNA component of *Saccharomyces cerevisiae* telomerase. *Science* 1994;266:404–409. [PubMed: 7545955]
35. Cohn M, Blackburn EH. Telomerase in yeast. *Science* 1995;269:396–400. [PubMed: 7618104]
36. Chen JL, Greider CW. An emerging consensus for telomerase RNA structure. *Proc. Natl Acad. Sci. USA* 2004;101:14683–14684. [PubMed: 15466703]
37. Fragnet L, Blasco MA, Klapper W, Rasschaert D. The RNA subunit of telomerase is encoded by Marek's disease virus. *J. Virol* 2003;77:5985–5996. [PubMed: 12719590]
38. Lingner J, et al. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 1997;276:561–567. [PubMed: 9110970] Describes the identification of TERT through a combination of ciliate extract biochemistry and yeast genetics studies.
39. Kelleher C, Teixeira MT, Forstemann K, Lingner J. Telomerase: biochemical considerations for enzyme and substrate. *Trends Biochem. Sci* 2002;27:572–579. [PubMed: 12417133]
40. Autexier C, Lue NF. The structure and function of telomerase reverse transcriptase. *Annu. Rev. Biochem* 2006;75:493–517. [PubMed: 16756500]
41. Collins K, Gandhi L. The reverse transcriptase component of the *Tetrahymena* telomerase ribonucleoprotein complex. *Proc. Natl Acad. Sci. USA* 1998;95:8485–8490. [PubMed: 9671704]
42. Lue NF, et al. Telomerase can act as a template- and RNA-independent terminal transferase. *Proc. Natl Acad. Sci. USA* 2005;102:9778–9783. [PubMed: 15994230]

43. Karamysheva Z, et al. Developmentally programmed gene elimination in *Euplotes crassus* facilitates a switch in the telomerase catalytic subunit. *Cell* 2003;113:565–576. [PubMed: 12787498]
44. Kilian A, et al. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. *Hum. Mol. Genet* 1997;6:2011–2019. [PubMed: 9328464]
45. Xiao S, Scott F, Fierke CA, Engelke DR. Eukaryotic ribonuclease P: a plurality of ribonucleoprotein enzymes. *Annu. Rev. Biochem* 2002;71:165–189. [PubMed: 12045094]
46. Blackburn EH. Telomerase and Cancer: Kirk A. Landon — AACR prize for basic cancer research lecture. *Mol. Cancer Res* 2005;3:477–482. [PubMed: 16179494]
47. Dong CK, Masutomi K, Hahn WC. Telomerase: regulation, function and transformation. *Crit. Rev. Oncol. Hematol* 2005;54:85–93. [PubMed: 15843091]
48. Blasco MA. Telomerase beyond telomeres. *Nature Rev. Cancer* 2002;2:627–633. [PubMed: 12154355]
49. Chang S, DePinho RA. Telomerase extracellular activities. *Proc. Natl Acad. Sci. USA* 2002;99:12520–12522. [PubMed: 12271146]
50. Weinrich SL, et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. *Nature Genet* 1997;17:498–502. [PubMed: 9398860] Establishes telomerase reconstitution *in vitro* using rabbit reticulocyte lysate.
51. Lingner J, Cech TR, Hughes TR, Lundblad V. Three ever shorter telomere (*EST*) genes are dispensable for *in vitro* yeast telomerase activity. *Proc. Natl Acad. Sci. USA* 1997;94:11190–11195. [PubMed: 9326584]
52. Feng J, et al. The RNA component of human telomerase. *Science* 1995;269:1236–1241. [PubMed: 7544491] Describes the human telomerase RNA and shows that it has many differences from ciliate telomerase RNAs.
53. Chapon C, Cech T, Zaug A. Polyadenylation of telomerase RNA in budding yeast. *RNA* 1997;3:1337–1351. [PubMed: 9409624]
54. Seto AG, Zaug AJ, Sobel SG, Wolin SL, Cech TR. *Saccharomyces cerevisiae* telomerase is an Sm small nuclear ribonucleoprotein particle. *Nature* 1999;401:177–180. [PubMed: 10490028]
55. Khusial P, Plaag R, Zieve GW. LSm proteins form heptameric rings that bind to RNA via repeating motifs. *Trends Biochem. Sci* 2005;30:522–528. [PubMed: 16051491]
56. Ferrezuelo F, Steiner B, Aldea M, Futcher B. Biogenesis of yeast telomerase depends on the importin mtr10. *Mol. Cell. Biol* 2002;22:6046–6055. [PubMed: 12167699]
57. Teixeira MT, Forstemann K, Gasser SM, Lingner J. Intracellular trafficking of yeast telomerase components. *EMBO Rep* 2002;3:652–659. [PubMed: 12101098]
58. Fu D, Collins K. Human telomerase and Cajal body ribonucleoproteins share a unique specificity of Sm protein association. *Genes Dev* 2006;20:531–536. [PubMed: 16481465]
59. Mitchell JR, Cheng J, Collins K. A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol. Cell. Biol* 1999;19:567–576. [PubMed: 9858580]
60. Meier UT. The many facets of H/ACA ribonucleoproteins. *Chromosoma* 2005;114:1–14. [PubMed: 15770508]
61. Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 1999;402:551–555. [PubMed: 10591218] Establishes the first connection between human telomerase deficiency and disease.
62. Mitchell JR, Collins K. Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase *in vivo* and *in vitro*. *Mol. Cell* 2000;6:361–371. [PubMed: 10983983]
63. Pogacic V, Dragon F, Filipowicz W. Human H/ACA small nucleolar RNPs and telomerase share evolutionarily conserved proteins NHP2 and NOP10. *Mol. Cell. Biol* 2000;20:9028–9040. [PubMed: 11074001]
64. Dragon F, Pogacic V, Filipowicz W. *In vitro* assembly of human H/ACA small nucleolar RNPs reveals unique features of U17 and telomerase RNAs. *Mol. Cell. Biol* 2000;20:3037–3048. [PubMed: 10757788]

65. Fu D, Collins K. Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. *Mol. Cell* 2003;11:1361–1372. [PubMed: 12769858]
66. Jady BE, Bertrand E, Kiss T. Human telomerase RNA and box H/ACA scaRNAs share a common Cajal body-specific localization signal. *J. Cell. Biol* 2004;164:647–652. [PubMed: 14981093]
67. Lingner J, Cech TR. Purification of telomerase from *Euplotes aediculatus*: requirement of a primer 3' overhang. *Proc. Natl Acad. Sci. USA* 1996;93:10712–10717. [PubMed: 8855245]
68. Aigner S, et al. *Euplotes* telomerase contains an La motif protein produced by apparent translational frameshifting. *EMBO J* 2000;19:6230–6239. [PubMed: 11080168]
69. Witkin KL, Collins K. Holoenzyme proteins required for the physiological assembly and activity of telomerase. *Genes Dev* 2004;18:1107–1118. [PubMed: 15131081]
70. Aigner S, Postberg J, Lipps HJ, Cech TR. The *Euplotes* La motif protein p43 has properties of a telomerase-specific subunit. *Biochemistry* 2003;42:5736–5747. [PubMed: 12741831]
71. Prathapam R, Witkin KL, O'Connor CM, Collins K. A telomerase holoenzyme protein enhances telomerase RNA assembly with telomerase reverse transcriptase. *Nature Struct. Mol. Biol* 2005;12:252–257. [PubMed: 15696174] Shows the biochemical function of a holoenzyme protein in the assembly of TER with TERT.
72. O'Connor CM, Collins K. A novel RNA binding domain in *Tetrahymena* telomerase p65 initiates hierarchical assembly of telomerase holoenzyme. *Mol. Cell. Biol* 2006;26:2029–2036. [PubMed: 16507983]
73. Smogorzewska A, de Lange T. Regulation of telomerase by telomeric proteins. *Annu. Rev. Biochem* 2004;73:177–208. [PubMed: 15189140]
74. Wang JC, et al. Dissociation of telomerase activity and telomere length maintenance in primitive human hematopoietic cells. *Proc. Natl Acad. Sci. USA* 2005;102:14398–14403. [PubMed: 16172394]
75. Lundblad V, Szostak JW. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* 1989;57:633–643. [PubMed: 2655926] Establishes a genetic approach for dissecting the mechanisms of telomerase-mediated telomere-length maintenance and ultimately represents the first identification of a telomerase protein.
76. Lendvay TS, Morris DK, Sah J, Balasubramanian B, Lundblad V. Senescence mutants of *Saccharomyces cerevisiae* with a defect in telomere replication identify three additional *EST* genes. *Genetics* 1996;144:1399–1412. [PubMed: 8978029]
77. Taggart AK, Zakian VA. Telomerase: what are the Est proteins doing? *Curr. Opin. Cell Biol* 2003;15:275–280. [PubMed: 12787768]
78. Hughes TR, Evans SK, Weilbaecher RG, Lundblad V. The Est3 protein is a subunit of yeast telomerase. *Curr. Biol* 2000;10:809–812. [PubMed: 10898986]
79. Seto AG, Livengood AJ, Tzfati Y, Blackburn EH, Cech TR. A bulged stem tethers Est1p to telomerase RNA in budding yeast. *Genes Dev* 2002;16:2800–2812. [PubMed: 12414733]
80. Zappulla DC, Cech TR. Yeast telomerase RNA: a flexible scaffold for protein subunits. *Proc. Natl Acad. Sci. USA* 2004;101:10024–10029. [PubMed: 15226497]
81. Fisher TS, Taggart AK, Zakian VA. Cell cycle-dependent regulation of yeast telomerase by Ku. *Nature Struct. Mol. Biol* 2004;11:1198–1205. [PubMed: 15531893]
82. Taggart AK, Teng SC, Zakian VA. Est1p as a cell cycle-regulated activator of telomere-bound telomerase. *Science* 2002;297:1023–1026. [PubMed: 12169735] Defines genetic requirements for physical interaction of telomerase and telomeres.
83. Stellwagen AE, Haimberger ZW, Veatch JR, Gottschling DE. Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. *Genes Dev* 2003;17:2384–2395. [PubMed: 12975323] Describes an interesting mechanism for yeast telomerase recruitment to non-telomeric chromosome ends.
84. Peterson SE, et al. The function of a stem-loop in telomerase RNA is linked to the DNA repair protein Ku. *Nature Genet* 2001;27:64–67. [PubMed: 11138000]
85. LaBranche H, et al. Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nature Genet* 1998;19:199–202. [PubMed: 9620782]

86. Ford LF, Suh JM, Wright WE, Shay JW. Heterogeneous nuclear ribonucleoproteins C1 and C2 associate with the RNA component of human telomerase. *Mol. Cell. Biol* 2000;20:9084–9091. [PubMed: 11074006]
87. Zhang QS, Manche L, Xu RM, Krainer AR. hnRNP A1 associates with telomere ends and stimulates telomerase activity. *RNA* 2006;12:1–13. [PubMed: 16373489]
88. Beernink HT, Miller K, Deshpande A, Bucher P, Cooper JP. Telomere maintenance in fission yeast requires an Est1 ortholog. *Curr. Biol* 2003;13:575–580. [PubMed: 12676088]
89. Snow BE, et al. Functional conservation of the telomerase protein Est1p in humans. *Curr. Biol* 2003;13:698–704. [PubMed: 12699629]
90. Reichenbach P, et al. A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr. Biol* 2003;13:568–574. [PubMed: 12676087]
91. Jady BE, Richard P, Bertrand E, Kiss T. Cell cycle-dependent recruitment of telomerase RNA and Cajal bodies to human telomeres. *Mol. Biol. Cell* 2006;17:944–954. [PubMed: 16319170]
92. Tomlinson RL, Ziegler TD, Supakordej T, Terns RM, Terns MP. Cell cycle-regulated trafficking of human telomerase to telomeres. *Mol. Biol. Cell* 2006;17:955–965. [PubMed: 16339074]
93. Zhu Y, Tomlinson RL, Lukowiak AA, Terns RM, Terns MP. Telomerase RNA accumulates in Cajal bodies in human cancer cells. *Mol. Biol. Cell* 2004;15:81–90. [PubMed: 14528011]
94. Cioce M, Lamond AI. Cajal bodies: a long history of discovery. *Annu. Rev. Cell Dev. Biol* 2005;21:105–131. [PubMed: 16212489]
95. Etheridge KT, et al. The nucleolar localization domain of the catalytic subunit of human telomerase. *J. Biol. Chem* 2002;277:24764–24770. [PubMed: 11956201]
96. Yang Y, Chen Y, Zhang C, Huang H, Weissman SM. Nucleolar localization of hTERT protein is associated with telomerase function. *Exp. Cell Res* 2002;277:201–209. [PubMed: 12083802]
97. Bachand F, Boisvert F-M, Cote J, Richard S, Autexier C. The product of the survival of motor neuron (*SMN*) gene is a human telomerase-associated protein. *Mol. Biol. Cell* 2002;13:3192–3202. [PubMed: 12221125]
98. Wong JM, Kusdra L, Collins K. Subnuclear shuttling of human telomerase induced by transformation and DNA damage. *Nature Cell Biol* 2002;4:731–736. [PubMed: 12198499]
99. Khurts S, et al. Nucleolin interacts with telomerase. *J. Biol. Chem* 2004;279:51508–51515. [PubMed: 15371412]
100. Harrington L, et al. Human telomerase contains evolutionarily conserved catalytic and structural subunits. *Genes Dev* 1997;11:3109–3115. [PubMed: 9389643]
101. Martin-Rivera L, Herrera H, Albar JP, Blasco MA. Expression of mouse telomerase catalytic subunit in embryos and adult tissues. *Proc. Natl Acad. Sci. USA* 1998;95:10471–10476. [PubMed: 9724727]
102. Liu K, Hodes RJ, Weng N. Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. *J. Immunol* 2001;166:4826–4830. [PubMed: 11290757]
103. Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, Dimmeler S. Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. *Mol. Cell. Biol* 2003;23:4598–4610. [PubMed: 12808100]
104. Akiyama M, et al. Nuclear factor- κ B p65 mediates tumor necrosis factor α -induced nuclear translocation of telomerase reverse transcriptase protein. *Cancer Res* 2003;63:18–21. [PubMed: 12517770]
105. Seimiya H, et al. Involvement of 14-3-3 proteins in nuclear localization of telomerase. *EMBO J* 2000;19:2652–2661. [PubMed: 10835362]
106. Fang G, Cech TR. Telomerase RNA localized in the replication band and spherical subnuclear organelles in hypotrichous ciliates. *J. Cell Biol* 1995;130:243–253. [PubMed: 7615628]
107. Mollenbeck M, et al. The telomerase-associated protein p43 is involved in anchoring telomerase in the nucleus. *J. Cell Sci* 2003;116:1757–1761. [PubMed: 12665556]

108. Ray S, Karamysheva Z, Wang L, Shippen DE, Price CM. Interactions between telomerase and primase physically link the telomere and chromosome replication machinery. *Mol. Cell. Biol* 2002;22:5859–5868. [PubMed: 12138196]
109. Lin J, Blackburn EH. Nucleolar protein PinX1p regulates telomerase by sequestering its protein catalytic subunit in an inactive complex lacking telomerase RNA. *Genes Dev* 2004;18:387–396. [PubMed: 14977919]
110. Zhou XZ, Lu KP. The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. *Cell* 2001;107:347–359. [PubMed: 11701125]
111. Banik SS, Counter CM. Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR. *J. Biol. Chem* 2004;279:51745–51748. [PubMed: 15381700]
112. Holt SE, et al. Functional requirement of p23 and Hsp90 in telomerase complexes. *Genes Dev* 1999;13:817–826. [PubMed: 10197982]
113. Forsythe HL, Jarvis JL, Turner JW, Elmore LW, Holt SE. Stable association of hsp90 and p23, but not hsp70, with active human telomerase. *J. Biol. Chem* 2001;276:15571–15574. [PubMed: 11274138]
114. Kim JH, et al. Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. *Genes Dev* 2005;19:776–781. [PubMed: 15805468]
115. Miller MC, Collins K. The *Tetrahymena* p80/p95 complex is required for proper telomere length maintenance and micronuclear genome stability. *Mol. Cell* 2000;6:827–837. [PubMed: 11090621]
116. Liu Y, et al. Telomerase-associated protein TEP1 is not essential for telomerase activity or telomere length maintenance *in vivo*. *Mol. Cell. Biol* 2000;20:8178–8184. [PubMed: 11027287]
117. Mason DX, Autexier C, Greider C. W; *Tetrahymena* proteins p80 and p95 are not core telomerase components. *Proc. Natl Acad. Sci. USA* 2001;98:12368–12373. [PubMed: 11592988]
118. Bateman A, Kickhoefer V. The TROVE module: a common element in telomerase, Ro and vault ribonucleoproteins. *BMC Bioinformatics* 2003;4:49. [PubMed: 14563212]
119. Macrae IJ, Doudna JA. Ro's role in RNA reconnaissance. *Cell* 2005;121:495–496. [PubMed: 15907458]
120. Ford LP, Shay JW, Wright WE. The La antigen associates with the human telomerase ribonucleoprotein and influences telomere length *in vivo*. *RNA* 2001;7:1068–1075. [PubMed: 11497426]
121. Le S, Sternglanz R, Greider CW. Identification of two RNA-binding proteins associated with human telomerase RNA. *Mol. Biol. Cell* 2000;11:999–1010. [PubMed: 10712515]
122. Mili S, Steitz JA. Evidence for reassociation of RNA-binding proteins after cell lysis: implications for the interpretation of immunoprecipitation analyses. *RNA* 2004;10:1692–1694. [PubMed: 15388877]
123. Collins K. Ciliate telomerase biochemistry. *Annu. Rev. Biochem* 1999;68:187–218. [PubMed: 10872448]
124. Lue NF. Adding to the ends: what makes telomerase processive and how important is it? *Bioessays* 2004;26:955–962. [PubMed: 15351966]
125. Jacobs SA, Podell ER, Cech TR. Crystal structure of the essential N-terminal domain of telomerase reverse transcriptase. *Nature Struct. Mol. Biol* 2006;13:218–225. [PubMed: 16462747] Reveals part of the TERT interaction site for single-stranded DNA at atomic resolution.
126. Theimer CA, Feigon J. Structure and function of telomerase RNA. *Curr. Opin. Struct. Biol.* 2006 May 16; (doi:10.1016/j.sbl.2006.05.005).
127. Lai CK, Miller MC, Collins K. Template boundary definition in *Tetrahymena* telomerase. *Genes Dev* 2002;16:415–420. [PubMed: 11850404]
128. Tzfati Y, Fulton TB, Roy J, Blackburn EH. Template boundary in a yeast telomerase specified by RNA structure. *Science* 2000;288:863–867. [PubMed: 10797010]
129. Chen JL, Greider CW. Template boundary definition in mammalian telomerase. *Genes Dev* 2003;17:2747–2752. [PubMed: 14630939]

130. Miller MC, Collins K. Telomerase recognizes its template by using an adjacent RNA motif. *Proc. Natl Acad. Sci. USA* 2002;99:6585–6590. [PubMed: 11997465] Shows that TERT can copy an exchangeable template but must co-assemble with non-template regions of telomerase RNA to acquire repeat-synthesis activity.
131. Benjamin S, Baran N, Manor H. Interference footprinting analysis of telomerase elongation complexes. *Mol. Cell. Biol* 2000;20:4224–4237. [PubMed: 10825187]
132. Baran N, Haviv Y, Paul B, Manor H. Studies on the minimal lengths required for DNA primers to be extended by the *Tetrahymena* telomerase: implications for primer positioning by the enzyme. *Nucl. Acids Res* 2002;30:5570–5578. [PubMed: 12490725]
133. Hardy CD, Schultz CS, Collins K. Requirements for the dGTP-dependent repeat addition processivity of recombinant *Tetrahymena* telomerase. *J. Biol. Chem* 2001;276:4863–4871. [PubMed: 11096070]
134. Lai CK, Mitchell JR, Collins K. RNA binding domain of telomerase reverse transcriptase. *Mol. Cell. Biol* 2001;21:990–1000. [PubMed: 11158287]
135. O'Connor CM, Lai CK, Collins K. Two purified domains of telomerase reverse transcriptase reconstitute sequence-specific interactions with RNA. *J. Biol. Chem* 2005;280:17533–17539. [PubMed: 15731105]
136. Lee SR, Wong JM, Collins K. Human telomerase reverse transcriptase motifs required for elongation of a telomeric substrate. *J. Biol. Chem* 2003;278:52531–52536. [PubMed: 14565961]
137. Lue NF. A physical and functional constituent of telomerase anchor site. *J. Biol. Chem* 2005;280:26586–26591. [PubMed: 15905172]
138. Moriarty TJ, Ward RJ, Taboski MA, Autexier C. An anchor site-type defect in human telomerase that disrupts telomere length maintenance and cellular immortalization. *Mol. Biol. Cell* 2005;16:3152–3161. [PubMed: 15857955]
139. Lai CK, Miller MC, Collins K. Roles for RNA in telomerase nucleotide and repeat addition processivity. *Mol. Cell* 2003;11:1673–1683. [PubMed: 12820978]
140. Friedman KL, Cech TR. Essential functions of amino-terminal domains in the yeast telomerase catalytic subunit revealed by selection for viable mutants. *Genes Dev* 1999;13:2863–2874. [PubMed: 10557213]
141. Armbruster BN, Banik SSR, Guo C, Smith AC, Counter CM. N-terminal domains of the human telomerase catalytic subunit required for enzyme activity *in vivo*. *Mol. Cell. Biol* 2001;22:7775–7786. [PubMed: 11604512]
142. Banik SSR, et al. C-terminal regions of the human telomerase catalytic subunit essential for *in vivo* enzyme activity. *Mol. Cell. Biol* 2002;22:6234–6246. [PubMed: 12167716]
143. Bryan TM, Goodrich KJ, Cech TR. *Tetrahymena* telomerase is active as a monomer. *Mol. Biol. Cell* 2003;14:4794–4804. [PubMed: 13679509]
144. Cunningham DD, Collins K. Biological and biochemical functions of RNA in the *Tetrahymena* telomerase holoenzyme. *Mol. Cell. Biol* 2005;25:4442–4454. [PubMed: 15899850]
145. Livengood AJ, Zaug AJ, Cech TR. Essential regions of *Saccharomyces cerevisiae* telomerase RNA: separate elements for Est1p and Est2p interaction. *Mol. Cell. Biol* 2002;22:2366–2374. [PubMed: 11884619]
146. Prescott J, Blackburn EH. Functionally interacting telomerase RNAs in the yeast telomerase complex. *Genes Dev* 1997;11:2790–2800. [PubMed: 9353249]
147. Wenz C, et al. Human telomerase contains two cooperating telomerase RNA molecules. *EMBO J* 2001;20:3526–3534. [PubMed: 11432839]
148. Marrone A, Stevens D, Vulliamy T, Dokal I, Mason PJ. Heterozygous telomerase RNA mutations found in dyskeratosis congenita and aplastic anemia reduce telomerase activity via haploinsufficiency. *Blood* 2004;104:3936–3942. [PubMed: 15319288]
149. Ly H, et al. Functional characterization of telomerase RNA variants found in patients with hematologic disorders. *Blood* 2005;105:2332–2339. [PubMed: 15550482]
150. Cerone MA, Ward RJ, Londono-Vallejo JA, Autexier C. Telomerase RNA mutated in autosomal dyskeratosis congenita reconstitutes a weakly active telomerase enzyme defective in telomere elongation. *Cell Cycle* 2005;4:585–589. [PubMed: 15753647]

151. Beattie TL, Zhou W, Robinson MO, Harrington L. Functional multimerization of the human telomerase reverse transcriptase. *Mol. Cell. Biol* 2001;21:6151–6160. [PubMed: 11509658]
152. Wong JM, Kyasa MJ, Hutchins L, Collins K. Telomerase RNA deficiency in peripheral blood mononuclear cells in X-linked dyskeratosis congenita. *Hum. Genet* 2004;115:448–455. [PubMed: 15349768]
153. Vulliamy T, et al. The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature* 2001;413:432–435. [PubMed: 11574891] The first report to link telomerase RNA mutations with human disease.
154. Vulliamy TJ, Marrone A, Dokal I, Mason PJ. Association between aplastic anemia and mutations in telomerase RNA. *Lancet* 2002;359:2168–2170. [PubMed: 12090986]
155. Vulliamy TJ, et al. Mutations in dyskeratosis congenita: their impact on telomere length and the diversity of clinical presentation. *Blood* 2006;107:2680–2685. [PubMed: 16332973]
156. Zahler AM, Williamson JR, Cech TR, Prescott DM. Inhibition of telomerase by G-quartet DNA structures. *Nature* 1991;350:718–720. [PubMed: 2023635]
157. Oganessian L, Moon IK, Bryan TM, Jarstfer MB. Extension of G-quadruplex DNA by ciliate telomerase. *EMBO J* 2006;25:1148–1159. [PubMed: 16511573]
158. Paeschke K, Simonsson T, Postberg J, Rhodes D, Lipps HJ. Telomere end-binding proteins control the formation of G-quadruplex DNA structures *in vivo*. *Nature Struct. Mol. Biol* 2005;12:847–854. [PubMed: 16142245]
159. Lansdorp PM. Major cutbacks at chromosome ends. *Trends Biochem. Sci* 2005;30:388–395. [PubMed: 15936947]
160. Wang H, Gilley D, Blackburn EH. A novel specificity for the primer-template pairing requirement in *Tetrahymena* telomerase. *EMBO J* 1998;17:1152–1160. [PubMed: 9463392]

Figure 1. Endogenous biogenesis pathways for telomerase holoenzymes

Schematic illustration of the events that accompany telomerase biogenesis *in vivo* through the following steps: telomerase RNA subunit (TER) expression, TER processing and assembly to form a stable telomerase ribonucleoprotein (RNP), and additional assembly to form a telomerase holoenzyme(s). **a** | In budding yeast, the TER precursor is transcribed by RNA polymerase II (Pol II) and gains a tract of polyadenosine (Poly A). It then undergoes assembly steps (possibly including heterogeneous nuclear (hn) RNP assembly and nuclear export) that result in the loading of a heteroheptameric ring of Sm proteins and the addition of the 5'-trimethylguanosine (TMG) cap. Holoenzyme form(s) of RNP also include the telomerase subunits Est1 and Est3 as well as telomerase reverse transcriptase (TERT) and the DNA-end-binding protein Ku. **b** | In human cells (vertebrates in the figure), the TER precursor is transcribed by Pol II. The nascent transcript is modified with a 5'-TMG cap and

binds to the H/ACA-motif proteins that direct 3'-end processing. Holoenzyme form(s) of RNP also include TERT. **c** | In *Tetrahymena thermophila* (ciliates in the figure), TER is transcribed by Pol III and binds to the telomerase subunits p65 and TERT. Holoenzyme form(s) of RNP also include telomerase p45 and p75.

Figure 2. Regulated engagement of telomerase with its chromosome substrates

Telomerase engages with telomeres in a dynamic manner that is governed by changes in the state of both interaction partners. The dark blue strand of DNA (middle left) represents guanosine-rich telomeric repeats and has the chromosome 3'-OH end, which must be positioned in the telomerase-holoenzyme active site for elongation. To reach this state, telomerase ribonucleoprotein (RNP) assembles with the telomerase reverse transcriptase (TERT), to generate a catalytically active enzyme, and with additional factors to ultimately form an enzyme that has activity on telomeres. This process seems to be affected by the sequestration of components in distinct subnuclear compartments. To be elongated by telomerase, telomeric chromatin is remodelled into a more accessible state and binds to factors that promote telomerase interaction. After each telomeric repeat synthesis, telomerase either repositions itself for processive repeat synthesis or it dissociates from the telomere. Dissociation might involve reversible disassembly or it might require the remodelling or degradation of the telomerase holoenzyme and/or the telomeric chromatin.

Figure 3. A multi-step catalytic cycle for telomeric repeat synthesis

The telomerase catalytic cycle is illustrated based on studies of the *Tetrahymena thermophila* holoenzyme¹⁶⁰. The telomerase RNA subunit (TER) template is shown with limited 5'- and 3'-flanking regions that anchor it in the vicinity of the active site; the template-adjacent single-stranded-DNA-binding site PAS1 (primer/product alignment/anchor site-1) is also shown (inset box). **1** | Template positioning at default is thought to place residue 49 of TER in the active site. **2** | Primer binding at PAS1 and 3'-end hybridization with the template can reposition the template from this default position. **3** | Elongation is proposed to occur such that up to five base-pairs of hybrid can form between the template and the product. **4** | Some hybrid dissociation occurs before the 5' end of the template enters the active site, (**5**) facilitating product release from the template. The free template can relax into the default position and (**2**) can re-engage a product 3' end for processive repeat synthesis.

Figure 4. Working model for TERT domain architecture

a | The telomerase reverse transcriptase (TERT) is divided into four regions, the TERT essential N-terminal (TEN) domain, the TERT high-affinity RNA-binding domain (TRBD), the reverse-transcriptase (RT) motifs and the TERT C-terminal (TEC) extension. **b** | A speculative illustration of the three TERT domains, TEN, TRBD and RT, that shows the coordination of the proposed sites of protein–nucleic-acid interaction in *Tetrahymena thermophila* telomerase. The template is shown with limited 5'- and 3'-flanking regions, as in FIG. 3. Single-stranded DNA that is adjacent to the template binds to PAS1 (primer/product alignment/anchor site-1), with additional PAS2 contacts in a subset of holoenzymes. At least part of PAS1 is located in the TEN domain, at least part of the 5'-template anchor site is located in the TRBD domain, and the active site is located in the RT domain. A 3'-template anchor site is modelled as located in the TEN domain, but the principles of 3'-template anchoring are not well understood. The TEN and TEC domains could both be part of the PAS2 (not shown; see text for discussion).