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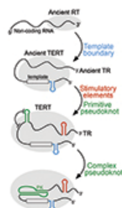

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REVIEW

# The role of telomeres and telomerase reverse transcriptase isoforms in pluripotency induction and maintenance

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**ABSTRACT**

Telomeres are linear guanine-rich DNA structures at the ends of chromosomes. The length of telomeric DNA is actively regulated by a number of mechanisms in highly proliferative cells such as germ cells, cancer cells, and pluripotent stem cells. Telomeric DNA is synthesized by way of the ribonucleoprotein called telomerase containing a reverse transcriptase (TERT) subunit and RNA component (*TERC*). TERT is highly conserved across species and ubiquitously present in their respective pluripotent cells. Recent studies have uncovered intricate associations between telomeres and the self-renewal and differentiation properties of pluripotent stem cells. Interestingly, the past decade's work indicates that the TERT subunit also has the capacity to modulate mitochondrial function, to remodel chromatin structure, and to participate in key signaling pathways such as the Wnt/$\beta$-catenin pathway. Many of these non-canonical functions do not require TERT's catalytic activity, which hints at possible functions for the extensive number of alternatively spliced TERT isoforms that are highly expressed in pluripotent stem cells. In this review, some of the established and potential routes of pluripotency induction and maintenance are highlighted from the perspectives of telomere maintenance, known TERT isoform functions and their complex regulation.

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## Background

The evolution of linearly organized DNA and inability of DNA polymerases to conserve the chromosomal ends have necessitated the need for non-coding telomeric DNA to cap the chromosomes.[1-3] These repeating DNA sequences protect an individual's chromosomes from fusion and erosion of coding DNA. Continued shortening of telomeric DNA due to the end-replication-problem eventually leads to critically short telomeres that induce senescence to avoid chromosomal damage.[3] This effect, known as the Hayflick limit [4,5] defines the number of possible cellular replications before cells senesce, and thus limits cellular lifespan. On an organismal scale, short telomere syndromes exhibit strong correlations with certain pathologies, such as pulmonary fibrosis,[6,7] arteriosclerosis,[8] aplastic anemia,[6,9] chronic liver disease,[10] and intrauterine growth restriction (IUGR) due to trophoblast stem cell dysfunction.[11-13]

Landmark work published by Carol Greider and Elizabeth Blackburn [14] uncovered the telomerase protein complex needed to re-lengthen telomeric DNA and maintain proliferation of a species and its germ cells. At the core of this ribonucleoprotein complex, the telomerase reverse transcriptase (TERT) protein in conjunction with its telomerase RNA component (*TERC*) is sufficient to extend telomeric sequences *in vitro*.[15] Elevated expression of TERT is found in over 90% of cancers in order to maintain proliferation, and much effort has been made in unveiling the pathogenic role of TERT in the diseased state (as previously reviewed [16,17]). However, high TERT levels naturally arise in non-pathogenic settings such as in the germline and stem cells, and in particular embryonic stem cells (ESCs). Prolonged proliferative capacity of ECSs is achieved by the maintenance/re-lengthening of telomere length via high telomerase activity. As such, human (h)ESCs are capable of being passaged over 120 times with no decrease in telomerase activity, telomere length and pluripotency potential.[18]

Several recent reviews [19-21] have highlighted different mechanisms by which telomeres and TERT are affected by pluripotency. These discussions tend to be singularly focused with pluripotency acting as an effector. Canonically, any feedback from TERT to pluripotency occurs only indirectly through telomere-mediated effects such as DNA damage responses (DDR). It is well established that telomere length (in particular critically short telomeres), as maintained through the canonical function of TERT, affects pluripotency[22] and embryonic viability.[23] However, the past decade's work on TERT has revealed non-canonical, extra-telomeric functions of TERT, much of whose functional significance are yet to be elucidated in the context of stem cell biology. It is quite possible that many of the non-canonical pathway functions of TERT do affect pluripotency and stem cell maintenance. Recent studies[24,25] have uncovered novel non-canonical functionality in some of the TERT isoforms that may also impact stem cell function. This review will discuss the multiple known and speculative connections to pluripotency induction and maintenance in the context of telomeres length, TERT isoforms, and their canonical and non-canonical functions.

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## Telomeres and pluripotent stem cells

Telomere length is a basic requirement for sustaining the replicative potential of somatic cells and its maintenance allows for unlimited self-renewal of stem cells.[18,26] Both murine and human embryonic stem cells demonstrate robust telomerase expression for extended population doublings and are capable of maintaining long telomeres.[27,28] Similarly, human and murine induced pluripotent stem cells (iPSCs) are capable of carrying out re-elongation of telomeres in at least some clones, although the telomere length and activity characteristics appear to be clonally unique.[28-33] Conversely, the process of differentiation triggers down-regulation of telomerase and initiates telomeric attrition.[34,35] Following the extended cellular division required to form adult tissues, terminally differentiated cell types typically exhibit low or undetectable levels of telomerase activity and shortened telomeres.[36,37] While extensive species similarities in the regulation of TERT and telomeres exist, Table 1 highlights some significant differences that will be highlighted in subsequent sections.

Both the increase and attrition of telomere length occurs gradually during S-phase [38] and requires multiple cell divisions. On the other hand, the initial stage of iPSC reprogramming is a fairly rapid process, and therefore telomere-mediated effects may not arise in the midst of reprogramming. Not surprisingly, the age of human cell donor has little effect on the ability to reprogram the cells to a pluripotent state.[30,39] In fact, human iPSCs (hiPSC) derived from cells with telomerase activity defects and non-critically short telomeres still can be reprogrammed to a pluripotent state.[29,40] This suggests that, while critically short telomeres significantly impact reprogramming efficiency, increasing telomere length far beyond the length needed to support initial proliferation requirements does not confer additional reprogramming advantage. This point is often masked by the use of mean telomere length, which generally has correlation with effects such as lifespan and pluripotency near short lengths where the telomere length distribution skewness is changing significantly.[41] However, following reprogramming mis-localization of human TERT (hTERT), and all abnormalities leading to similar telomere attrition result in eventual loss of self-renewal and pluripotency.[42] Similarly, long term self-renewal and teratoma formation in murine and porcine iPSCs with short telomeres are generally impaired.[31,43] In addition, PSCs with short telomeres in a variety of species display mitochondrial dysfunction,[44] impaired neuroectodermal differentiation,[44] uncapping events, and reduction in H3K27me3 distal to telomeres including global hypomethylation which contributes to reactivation of pluripotency genes,[45] and possibly reprogramming transgenes.[31,33,46]

Partially reprogrammed iPSCs often show little TERT activation and short telomeres.[32] This reprogramming failure is likely the reason for the failure of the TERT promoter to be activated. Conversely, it is unlikely that lack of TERT activation causes partial reprogramming as TERT expression and/or activity does not guarantee pluripotency.[28,35,47,48] However, telomere length is touted as a biomarker of reprogramming, with hiPSC telomere lengths plateauing close to the hESC telomere length of about 12 kbp despite continued high telomerase expression.[49] Such stabilization of telomere length, in addition to proper downregulation of hTERT following differentiation, is a strong hallmark of non-transformed human pluripotent stem cells.[30,35,49] hESCs and hiPSCs normally suppress the ALT (alternative lengthening of telomerase) pathway[49] and, in the absence of hTERT, short telomeres typically lead to up-

**Table 1.** Species-specific comparison of telomere/telomerase effects and their interactions with pluripotency regulation.

| | | Organism | | |
|---|---|---|---|---|
| | | **homo sapiens** | **murine** | **porcine** |
| **Telomere Length** | **Inner cell mass** | 12 kb [162] | 86 kb in-vivo 50 kb in-vitro [52] | |
| | **ESCs** | 12 kb [49] | 144 kb at p12 [52] | 33.0[46] |
| | **iPSCs** | Similar to ES[49] | similar to ES[53] | up to 31.5kb[31] |
| **Effect on ES/iPSC pluripotency** | **TERT *knockdown**** | No[86]/Yes [84,85] | No [45,124] /Reprogramming effects [63] | unknown |
| | ***TERC knockdown**** | No[40,85] | No major effect [51]/mitochondria [44], naivety [33] | unknown |
| **TERT Null Phenotype** | **TERT−/−*** | unknown/unknown | None[76,83,123], /WNT-defect [105] | unknown |
| | ***TERC*−/−*** | unknown/unknown | None [77] | unknown |
| **iPSC Reprogramming** | **Telomeres re-lengthen** | Yes-sporadic [28] | Yes-sporadic [32] | Yes-sporadic[31] |
| | **Elongation period** | up to passage 15 [49] | continuous [52] | unknown |
| | **ALT pathway** | supressed [49] | active [46] | active[31] |
| **iPSC TERT increase** | | >100x [32] | approx. 3x [32] | |
| **Knockdown SUV39h1 and SUV39h2**[46] | | Telomere shortening | Telomere elongation | Telomere elongation |
| **Non-telomere Interactions** | **NF-kB (RelA/p65)** [111,163] | Stabilizes p65 and its Nuclear Residency<br>IL-6,8 & TNF-a promoter binding | mTERC−/− and mTERT−/− defective NF-kB signaling | |
| | **BRG1** [111,105] | BRG1/GNL3 | BRG1 | |
| | **RNA species** [102,107] | RMRP<br>LINE1/alphoid ssRNA | | |
| | **Mitochondrial membrane proteins** [158] | TOM20/TOM40 & TIM23 | | |
| | **mtDNA** [158] | ND1 & ND2 mtDNA | | |

Categories with mixed results are indicated with supporting references.
*in absence of short telomere effects.

regulation of the p53-mediated apoptosis pathway, or chromosomal rearrangements.[29] Thus, the aforementioned telomere maintenance characteristics in pluripotency are not preserved across vertebrates (Table 1).

Instead of using murine TERT (mTERT), initial lengthening of murine telomeres during early embryo cleavage[50] is largely achieved through alternative lengthening of telomerase (ALT) pathways. To a lesser extent, mESCs and miPSCs also appear to upregulate and utilize ALT mechanisms for telomere maintenance intermittently.[33,51] mESCs and miPSCs also do not exhibit the *in vitro* plateau in telomere length that is characteristic of hESCs/hiPSCs.[52] While NT-mESCs generally have completed telomere lengthening and full reprogramming by the blastocyst stage,[44] miPSCs may take up to 30 passages to achieve mESC lengths [53] and only do so sporadically.[32] Control of telomere length is an intricate process involving many factors. As previously reviewed,[19] mESCs and miPSCs control telomere length largely by modulating telomeric epigenetics and regulation by several proteins, including ZSCAN4,[51,54-56] ATRX,[54,55] RIF1,[56] TRF1[52,57] TPP1 and other shelterin complex components.[52,58] Increased telomere length, as part of the establishment of the pluripotent epigenome, occurs only after multiple passages, and is accomplished through epigenetic modifications of histones and subtelomeric DNA methylation.[33,59] During cellular reprogramming, hiPSC sub-telomeres become hyper-methylated with both *de novo* methylation and pockets of demethylation occurring.[34] At the same time, histone H3.3 plays a critical role in regulating telomeric chromatin accessibility.[60] Whereas during differentiation H3.3 is decreased causing telomeres and subtelomeres to take on a more heterochromatic state, complete knockdown of H3.3 leads to telomeric dysfunction.[60] Early lengthening of telomeres, within the first few passages following reprogramming, is preceded by a significant reduction of H3K9/H4K20 trimethylation.[49] However, this must be followed by reestablishment of H3K9/H4K20 me3 repressive marks to stabilize telomeric length.[49]

While knockdown of histone methyltransferases (HMTs) SUV39h1 and SUV39h2 in mice and pigs leads to increased telomere length, decreased demethylases DNMT1/3a/3b and decreased H3K9me3 marks,[46] knockdown in human cells leads to telomere length shortening.[46] This disparity is likely due to species differences (Table 1) that repress ALT pathways in humans, but not murine or porcine cells.[31,46] Consequently, although heterozygous mTERC−/+ miPSCs and to a lesser extent mTERC−/− miPSCs are capable of maintaining pluripotency and telomere length in mice, possibly due to the activation of the ALT pathway,[33] hTERT−/+ hiPSCs show poor telomere elongation, and DKC1 (Dyskerin - a telomerase complex component) hiPSC mutants (TERC deficient) do not elongate telomeres.[42]

## Pluripotency and canonical TERT functions

As discussed above, telomeres have strong feedback mechanisms to regulate pluripotency. TERT is the catalytic component of telomerase whose function, when combined with *TERC*, is to maintain telomere length and structure. This section will establish the canonical pluripotency-to-TERT links

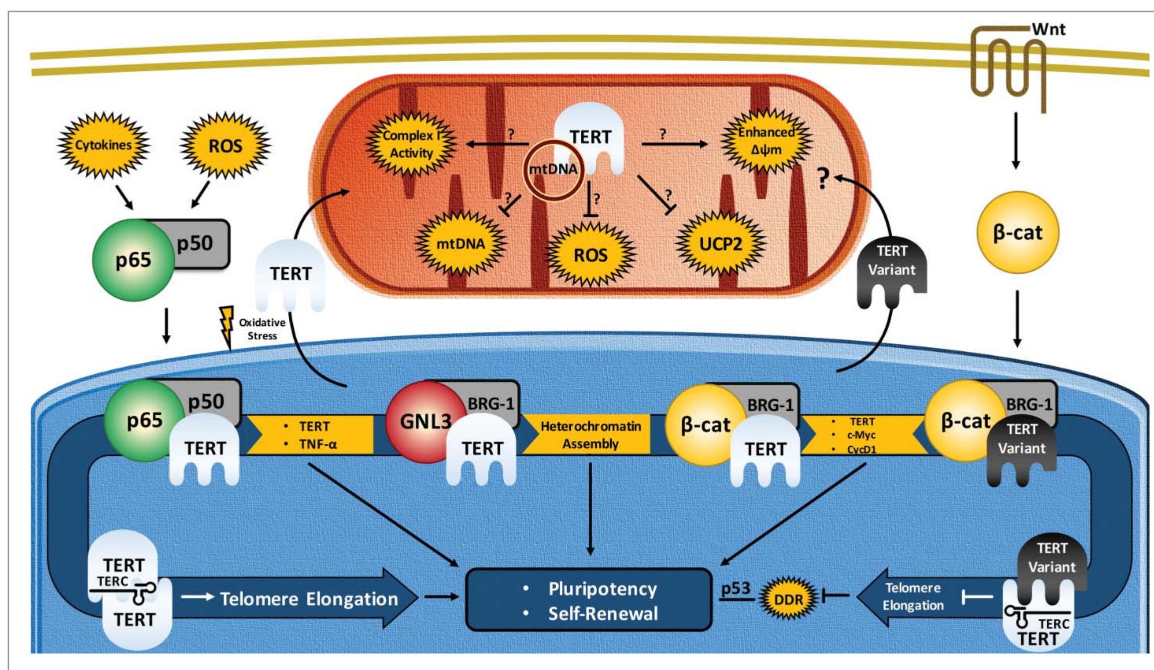


**Figure 1.** Proposed routes of pluripotency maintenance and self-renewal by pluripotent stem cells through functions of telomerase reverse transcriptase (TERT) isoforms. TERT expression is regulated by a number of key pluripotency/transcription factors. Conversely, TERT regulates pluripotency and self-renewal via canonical (telomeric) and non-canonical (mitochondrial, transcriptional) functions in pluripotent stem cells (PSCs). Telomere- and chromosome-mediated TERT effects lead to changes in pluripotency and cell proliferation by modulating the activities of transcription factors (c-Myc, $\beta$-Catenin, NF-$\kappa$B, p53). This figure would be much more complex with additional TERT variant specific functions, as well as multiple TERT splice variants modulating each of its current roles. Common acronyms: mtDNA (mitochondrial DNA), $\Delta\psi$m (Mitochondrial Membrane Potential/MMP), ROS (Reactive Oxygen Species) UCP2 (Mitochondria uncoupling protein 2), $\beta$-Cat ($\beta$-Catenin/CTNNB1), DDR (DNA Damage Response). The line without arrowhead underneath p53 indicates existing evidence for interaction with lack of mechanistic understanding.

that will be contrasted with the subsequent non-canonical TERT section (below). Putative links are highlighted in Fig. 1.

During embryogenesis and induced pluripotent stem cell (iPSC) generation, TERT and other telomerase components are upregulated, and remain so in self-renewing PSCs. TERT activation is a beneficial event for lengthening telomeres in order to stabilize them and suppressing the DNA damage response (DDR) prior to other epigenetic changes requiring extensive proliferation. Telomerase activity may thus be useful in assessing iPSC reprogramming efficiency and as a hallmark of non-transformed somatic cells, which should contain low telomerase expression and activity.[61] Moreover, upregulation of pluripotency genes is strongly connected with expression of TERT, the overexpression of which enhances reprogramming efficiency.[62,63] For example, porcine iPSCs can only maintain telomeres with continued expression of exogenous reprogramming factors to maintain TERT levels,[31] and partially reprogrammed mouse embryonic fibroblasts (MEFs), and porcine embryonic fibroblasts (PEFs) show little *TERT* promoter activation and poor telomere elongation. However, while low telomerase activity is correlated with partial reprogramming, high levels of TERT alone does not induce a pluripotent state,[48] nor does pluripotency strictly require high levels of TERT.[28] The transcriptional control by which TERT is upregulated during reprogramming to a pluripotent state has only recently begun to be elucidated.

During reprogramming, endogenous TERT up-regulation is a late event[64] that precedes endogenous upregulation of OCT4, SOX2 [33] and *TERC* (whose promoter is bound by OCT3/4 and NANOG [40]). TERT up-regulation is instead simultaneous with the overexpression of KLF4.[33] Recently, KLF4 binding has been mapped directly to the *TERT* proximal promoter where KLF4 is able to upregulate TERT when $\beta$-catenin acts as a cofactor.[49,65,66] This appears to be a key function of KLF4, as hTERT overexpression is capable of rescuing KLF4 knockdown-triggered cellular differentiation.[66] Furthermore, KLF4-$\beta$-catenin in complex with TCF-4 or TCF-1 serves either to activate or to repress TERT, respectively.[65,67] This interplay helps to provide a mechanism by which pluripotent and cancer stem cells are able to upregulate TERT to initiate cellular immortalization through telomere maintenance. Additionally, cell lines containing short telomeres are quite refractory to reprogramming and this inhibition is mediated by the p53 apoptosis/senescence pathway, which when removed allows the reprogramming of cell lines with critically short telomeres (albeit resulting in widespread chromosomal aberrations).[68] Hence, TERT upregulation through KLF4 may serve as an additional indirect means by which the p53 pathway can be suppressed via telomere maintenance during reprogramming.[69]

C-MYC (one of the original Yamanaka factors [70]) remains a common, albeit dispensable, cofactor during iPSC reprogramming,[71] and maintains high expression following transformation to a pluripotent state. C-MYC binds to and activates the *TERT* promoter.[65,72,73] Indirect evidence of C-MYC's importance is provided by the knockdown of SIRT1 which represses C-MYC, and accompanies a reduction in mTERT levels.[47] However, C-MYC is not required for reprogramming and its expression level is generally not correlated with TERT levels post-reprogramming, raising the question of whether C-MYC actually plays a major role in maintaining TERT expression.[32,33,53] It is possible that this effect is still mediated through KLF4 as SIRT1 also affects NANOG expression, which, in turn can regulate KLF4 and C-MYC through feedback loops with other pluripotency factors (for example OCT3/4 and mir145).[74,75] C-MYC may have a more important role in hiPSC generation where ALT does not contribute to the initial elongation of telomeres, and thus hTERT must play a more substantial role.

While non-canonical extra-telomeric functions of TERT are becoming apparent (*see* following section), it appears that, at least in the murine context, it is the canonical telomeric maintenance that has the greatest effect *in vitro*.[76,77] In agreement with this, complete abolition of telomerase function through mTERT [76] or *mTERC* [77] knockout in mice does not result in gross pathological symptoms for 6 to 8 generations at which point telomeres are critically shortened. Moreover, haplosufficient *mTERC*+/− mESCs can contribute to tetraploid embryos, whereas full-knockout *mTERC*−/− cells are unable to contribute despite containing unmodified mTERT.[78] Collectively, it can be deduced that TERT and canonical telomerase activity is dispensable during the reprogramming process of murine cell lines with sufficient telomere length,[33] but ultimately telomere maintenance is a required feature of prolonged self-renewal. In support of this, knockdown of ZSCAN4, an enhancer of chromosome stability and telomere-sister chromatid exchange (T-SCE)[51] initiates cell crisis and apoptosis within 8 passages in mESCs, whereas *mTERC*−/− cells upregulate ZSCAN4 possibly to compensate and are subsequently sustainable for up to 450 population doublings.[51]

Similarly, mTERT appears to be dispensable for the *in vitro* differentiation of mESCs with sufficient telomere length.[45] In a study by Pucci *et al.*, mTERT −/− mESCs with both short and long telomeres appear to differentiate normally upon LIF withdrawal.[45] However, differentiated cells derived from short telomere mESCs do not maintain their non-pluripotent status and begin to re-express NANOG [45] through unknown mechanisms. Prior to differentiation NANOG is also expressed at high levels in mTERC −/− mESCs with short telomeres.[44] Additionally, Pucci *et al.* found that ESRRB, a KLF4 substitute during reprogramming [79] and a direct target of NANOG,[80] is also strongly upregulated.[45] Previous studies have demonstrated p53 binding to the promoters of NANOG and ESRRB [81] and its ability to either enhance or repress the pluripotency genes.[80] Thus, p53 signaling in response to short telomeres of mTERT−/− mESCs may act to upregulate NANOG. Alternatively, NANOG up-regulation could be an incidental effect of DNA methyl transferase (DNMT) down-regulation leading to hypo-methylation in an attempt to upregulate the ALT pathway as previously proposed.[58] Another possibility is that the short telomeres might have led to destabilization of shelterin complexes and the resultant release of RAP1. RAP1 can modulate the steady state level of E-cadherin and consequently OCT3/4 and NANOG.[82] Lastly, as DKC1 regulates promoters of pluripotency genes, it is possible that removal of TERT from the telomerase complex disrupts the holoenzyme allowing DKC1 to more efficiently modulate pluripotency promoters.[83] Further investigation of mechanisms of telomeric induced deregulation of pluripotency

genes during differentiation should yield important information as to how short telomeres influence distal genes.

Little work has been carried out with the knockout/knockdown of telomerase subunits in human cells. Of the studies that have been carried out, conflicting results have emerged. Yang *et al.* [84] suggest that lentiviral knockdown of hTERT in hESCs results in their spontaneous differentiation and the inability to generate a stable ES cell line. In partial agreement, chemical inhibition of hTERT activity in hESCs leads to lowered pluripotency and increased differentiation, while curiously, similar blocking of telomerase activity through *TERC* steric inhibition has no effect suggesting an extra-telomeric role for TERT in maintaining self-renewal in the undifferentiated state.[85] In contrast, Sexton *et al.* did not observe any reduction in pluripotency (as measured by sustained OCT3/4 expression only) after establishing stable hESC lines through zinc finger-mediated knockout of hTERT.[86] However, *Sexton et al.* do observe a loss of self-renewal and differentiation beginning around 75 days post TERT-knockout, when the cells reach a critical telomere length of 2–3kb. These drastic differences likely arise from effects of the modalities used to knockout/knockdown hTERT or alternatively from telomeric differences in the cell lines used. Despite this, as discussed in the previous section (also *see* Table 1), humans do not normally have a substantial ALT pathway contribution and other species-specific differences exist. For example, mTERT expression is only upregulated at low (3 fold) levels during reprogramming as compared to hTERT in hiPSCs (several hundred fold), despite high promoter activation that correlates with reprogramming stage.[32] *TERC* is typically expressed in excess, even in differentiated tissues,[58] and thus may not be a limiting factor in *mTERC* +/− murine cells. Lastly, as laboratory mice are known to have extremely long telomeres and short lifespans, telomeric effects may be delayed compared to humans.

## Non-canonical functions of telomerase and pluripotency

In recent years, the discovery of telomere-independent effects of TERT on proliferation[87-95] and perturbations of resistance to reactive oxygen species damage [96-100] have led to a burgeoning body of evidence that indicates that TERT has many functions outside it's canonical role in telomere length maintenance.[101] Some of these extra-telomeric effects include acting as an RNA-dependent RNA polymerase,[102] a mitochondrial reverse transcriptase,[103] interacting with nucleostemin (GNL3),[104] WNT, BRG1, $\beta$-catenin,[87,105] NF-$\kappa$B,[106] and modulating endogenous siRNA [102,107] and miRNA profiles.[108] Although the non-canonical effects have been observed mainly in non-pluripotent cell types, many of the affected pathways play key roles in pluripotency. This section will discuss extra-telomeric roles and possible direct or indirect connections to pathways also involved in the self-renewal and maintenance of stem cells. Some of these confirmed and speculative pathways are summarized in Fig. 1.

### *TERT, BRG1 and Wnt*

A number of effects point to TERT (with or without *TERC*) possessing non-canonical functions. TERT is capable of aiding the maintenance of self-renewal in human limbal and mesenchymal stem cells, increasing the efficiency of directed differentiation,[48,99] enhancing iPSC reprogramming efficiency [40] and activating resting hair follicle stem cells.[90] Several recent reports have shown that TERT is able to interact with BRG1 in a WNT/$\beta$-catenin dependent manner. The canonical WNT pathway is extensively involved in differentiation of cell lineages and BRG1 loss of function is embryonic lethal and a critical developmental transcription factor.[109-113] Despite demonstrating TERT-BRG1 interaction in both murine cellular and tissue ChIP assays,[105] and the TERT-BRG1-GNL3 complex in specific human cell lines,[107] the widespread applicability of TERT-BRG1 interactions has been called into question.[114] Of 4 cell lines investigated, Listerman *et al.* could not show a consistent interaction,[114] and other studies have not found BRG1 interactions.[106,115] However, supportive evidence has been offered in several other studies.[105,107,108] MicroRNA (miRNA) expression alterations during the suppression of TERT in THP-1 and HeLa cells are strikingly similar to microRNA changes during BRG1 suppression.[108] Though mostly inferred from correlative data, it is likely that these changes are mediated through TERT's interaction with transcriptional factors BRG1, GNL3 and NF-$\kappa$B that regulate miRNAs and not by direct miRNA interaction.[108] Additionally, axis formation defects following TERT knockdown in *Xenopus* are consistent with a role for TERT in the Wnt pathway.[105] In contrast, in human cells, association of GNL3 with TERT-BRG1 produce RNA dependent RNA polymerase activity (RdRP), forming siRNA targeted to regulate centromeric alphoid domains, and non-centromeric LINE1 elements, thereby modulating heterochromatin during mitosis.[107] Thus, TERT-BRG1 may play varying roles depending on which cofactors are present, and these associations if present during pluripotency and differentiation would directly link TERT to a developmental role.

Unfortunately, despite *in vitro* evidence for TERT-BRG1 interactions, *in vivo* defects observed in *TERT*-null mice (with sufficient telomeres) have only been seen in a single mouse strain by one research group.[105] It is possible that interaction may be strongly context dependent and pathological transformed lines may have additional, or conversely lack, compensatory mechanisms for TERT-BRG1 interactions. On the other hand, multiple groups have indicated possible TERT interaction with $\beta$-catenin in both humans and mice [87,105,115] and the ability to activate resting hair and epithelial stem cells lends credence to an *in vivo* interaction possibly involving $\beta$-catenin.[87,90] Since even catalytically inactive TERT is able to enhance iPSC reprogramming [63] requiring an mesenchymal-to-epithelial transition (MET), it is initially curious that the TERT-$\beta$-catenin association supports the reverse epithelial-to-mesenchymal transition (EMT) in transformed cells.[115] The upregulation of the EMT inhibitor KLF7 in TERT containing iPSCs [63] and nuclear export of $\beta$-catenin by E-cadherin [116] may mitigate this effect . However, recent evidence suggests that early activation of an EMT pathway prior to a delayed EMT event actually enhances reprogramming.[117] Ultimately, the enhancement of iPSC reprogramming may be supported by more than one of TERT's non-canonical effects, such as enhanced proliferation, ROS mitigation, NF-$\kappa$B interaction in addition to its canonical telomere stabilizing effect.

### *TERT and mitochondria*

Mitochondrial localization of TERT is extensively documented, however, its role in mitigating ROS (Reactive Oxygen Species), and acting as a *TERC* independent reverse transcriptase are only beginning to be understood.[96-100,118,119] It is difficult to unravel the contributions from non-canonical TERT effects versus telomeric effects. The canonical telomere shortening effects generally occur after extended proliferation, and are mediated by telomeric dysfunction. On the other hand, non-canonical effects of TERT should appear immediately upon knockout. For example, removal of the canonical function of mTERT by *mTERC* knockout leads to mitochondrial dysfunction after iPSC and SCNT reprogramming of cells with short telomeres.[44] However, this mitochondrial dysfunction is likely a p53 mediated effect due to the poorly maintained telomeres. The donor cells came from late-generation Terc−/− mice (hence short telomeres to begin with) and were allowed to grow/differentiate under telomerase-activity-deficient conditions, which may have led to mitochondrial damage.[44] As there is evidence that most mitochondrial localized TERT may not carry *TERC* at all and instead act as a reverse transcriptase for mitochondrial RNA [103] it would be informative to additionally knockdown mTERT [44] to look for decreased ROS scavenging.

To add to the complexity, TERT is capable of binding alternative RNA's including the RNA component of mitochondrial RNA processing endoribonuclease *(RMRP)*, with which it can form dsRNA that is processed into siRNA directed against RMRP.[102] RMRP is required for development, and aside from its involvement in mitochondrial DNA replication,[120] it is also processed into siRNAs that targets several developmentally relevant genes.[121,122] TERT also appears capable of altering miRNA levels, possibly through a BRG1 associated mechanism,[108] although in rat cardiomyocytes the effect is solely on mature miRNAs and seems likely to be mediated through non-specific effects such as increased ROS.[123] It should be interesting to further investigate if these RMRP related effects are present in pluripotent stem cells and whether they are capable of altering cellular differentiation pathways.

Aside from the highlighted telomeric effects, TERT and *TERC* knockouts are not lethal in mice, nor do they result in phenotypic/transcriptional WNT pathway defects [124] or typical RMRP-related defects.[124] The extensive homology of TERT across species and in-particular vertebrates, and the ability to make at least one *hTERT*-null pluripotent human line [86] makes it difficult to imagine embryonic lethality in *hTERT*-null humans. On the other hand, as discussed in the previous sections, species differences exist and humans appear to suppress ALT pathways and rely more heavily on hTERT during development.[49] Alternatively, it is feasible that extra-telomeric effects may be redundant, appearing in sporadic situations where compensatory mechanisms are absent. As functional redundancy may, on a much larger scale, play a significant role in survival of a species, these effects still warrant a significant amount of attention. Additionally, as TERT has abundant isoforms, most of which are catalytically inactive at telomeres, these extra-telomeric roles may be significant.

## Alternative splice forms of telomerase

Telomerase expression and activity is highly regulated by a number of mechanisms, one of which is alternative splicing.[125-129] Alternative splicing is a method utilized by most mammalian cells to increase the transcriptome and protein diversity for extra means of gene regulation.[130] To date, 22 and 31 unique alternative splice variants of TERT have been identified for human and chicken, respectively.[25,131-134]

The human TERT gene is approximately 42 kb in length and contains 15 introns and 16 exons.[135] In most cases, exons are assembled together by joining of evolutionarily conserved 5′-splice donor and 3′-splice acceptor sequences in introns through long-range RNA interaction.[136] Alternative splicing of TERT is achieved by exon-skipping, intron-retention, and use of alternate splice acceptor sequences,[136] and is regulated by SRSF and hnRNP protein families.[24] However, the mechanism of regulation remains largely unclear. Notably, multiple splicing events can occur simultaneously in a single transcript, resulting in very diverse population of TERT isoforms.[137] In many cases, early splicing events within 5′-region of the transcript will introduce premature termination codons (PTCs) nullifying the effect of splicing 3′ to these,[25] causing many protein isoforms to be degenerate and/or targeted for non-sense mediated decay (NMD).[138] TERT has several evolutionarily conserved functional domains. The N-terminal end harbours a mitochondrial targeting sequence (MTS), nuclear localization signals (NLSs), and RNA-interacting domains, while the C-terminal half contains multiple reverse-transcriptase-like (RT-like) motifs.[135,139-145] These critical elements are often disrupted in a majority of TERT isoforms to varying extents.[25] Due to this, no currently identified alternative splice variant of hTERT exhibits reverse-transcriptase activity and only the full-length hTERT (containing all 16 exons) retains catalytic activity.[25] Nevertheless, catalytically inactive isoforms are highly expressed throughout human development in a tissue-specific and development-stage dependent manner.[126,128] Moreover, some hTERT isoforms are constitutively generated independently of the full-length hTERT levels.[24,125,127,128,146] Accumulating evidence points to potential roles of hTERT isoforms in the regulation of telomerase functions.[24,128,147-151]

An early attempt to characterize hTERT revealed two of the best-characterized hTERT isoforms, $\alpha$- and $\beta$-variants.[131,135] The $\alpha$-variant results from a partial in-frame deletion (36bp) of exon 6, leading to the partial loss of RT-motif A. This catalytically inactive form of hTERT is a dominant negative inhibitor of telomerase activity when overexpressed in telomerase positive immortal cells.[137,152] Considering the relatively low transcript abundance of the $\alpha$-variant,[146,147,151] the level of inhibition may be insufficient for it to have significant impact on telomere maintenance of indefinitely dividing cells. Shortly after the discovery of the $\alpha$-variant, Hisatomi *et al.* discovered a new in-frame splicing event that deletes two RT-motifs, rendering catalytic inactivity.[132] Like the $\alpha$-variant, the $\gamma$-variant was suggested as a negative regulator of telomerase activity. However, $\gamma$-transcript levels, relative to those of $\alpha$- and $\beta$-variants, are extremely low or nearly undetectable in tested cell types.[132,150] Despite low expression levels, the $\alpha$- and $\gamma$-variants hold much promise in micro-regulation of non-canonical

TERT functions, as they are both in-frame deletion variants with good structural and sequence homology to full-length TERT.

The $\beta$-splice variant results from a 182 bp out-of-frame deletion and the subsequent loss of exons 7 and 8.[131,135] Unlike the $\alpha$-deletion, the $\beta$-deletion introduces a premature termination codon (PTC) causing it to be truncated and a potential NMD target.[138] However, Listerman *et al.* and our group have recently shown that the $\beta$-variant transcript associates with polyribosomes, escapes NMD, and is likely translated.[24,85] Surprisingly, the $\beta$-deletion transcript not only survives degradation but is one of the most highly expressed TERT transcripts in various cell types including human embryonic stem cells.[24,128,132,147,151] A recent discovery suggests that the $\beta$-variant inhibits telomerase activity by sequestering *TERC*.[24] However, *TERC* is expressed at very high levels in most immortal cell types and is rarely a limiting factor for telomerase activity.[125,134,153,154] Despite the truncation, the $\beta$-variant retains the MTS, RNA-interaction sites and NLSs. Its abilities to interact with different RNA species and translocate between different cell compartments [24] are strongly suggestive of possible roles in regulation of RNA-mediated extra-telomeric functions of hTERT. Maida *et al.* was the first group to have detected RdRP activity of full-length TERT and to have characterized catalytically active hTERT as a negative regulator of RMRP via siRNA mediated-knockdown.[102,122] This was later confirmed by work of Mukherjee *et al.* in which they also observed reduced proliferation of human mammary epithelial cells upon shRNA mediated-RMRP knockdown.[155] Since the production of siRNA by TERT-RMRP requires catalytic activity, any catalytically inactive forms of hTERT with an intact RNA-binding domain such as $\beta$-variant may inhibit cell proliferation by down-regulating siRNA production. Overexpression of the $\beta$-variant in HeLa cells showed its ability to associate with mitochondria, the significance of which is not fully understood.[24] In the same study, overexpressed $\beta$-variant exhibited anti-apoptotic effects in cisplatin-treated human breast cancer cells by significantly reducing caspase 3/7 activity. Human TERT overexpression studies have shown that full-length hTERT augments mitochondrial function and maintains the integrity of mitochondria DNA under oxidative stress in a context dependent manner.[156-158] Remarkably, overexpression of dominant-negative mutant TERT abrogated or reversed some of these mitochondrial changes, pointing to a potential counter-regulatory role of hTERT alternative splice variants against full-length hTERT in regulating mitochondrial function.[158] This putative interplay between hTERT isoforms hold much significance in settings such as embryonic stem cells where mitochondria (and metabolism) likely play critical roles in governing the stress response, cell fate decisions, and pluripotency. Known factors that affect hTERT-splicing patterns are TGF-$\beta$1 and hypoxia.[159,160]

TGF-$\beta$1 in human epidermal cells shifts hTERT splicing patterns toward $\beta$-variant transcript at the expense of the full-length transcript,[159] whereas hypoxic conditions causes it to shift in the other direction.[160] TGF-$\beta$1 is an important cytokine involved in cell proliferation, stem cell maintenance and differentiation through SMAD signaling.[1,2] In addition, hypoxia increases hESC resistance to spontaneous differentiation and improves cell pluripotency through stabilization of hypoxia-inducible factors and the expression of their target genes.[161] Together, this data suggests that the preferential shift of the TERT splicing pattern is a highly regulated process, which can promptly respond to extra-cellular stimuli that ultimately affect stem cell identity. Reverse-transcriptase function of mTERT may be dispensable for modulation of Wnt-$\beta$-catenin signaling and its targeted gene expression.[105] This indicates the likelihood that naturally occurring catalytically inactive TERT isoforms regulate Wnt signaling possibly through disruption and/augmentation of the Brg1-TCF-TERT transcription complex formation.

More recently, a novel hTERT isoform (now termed Δ4–13 variant) was discovered in both telomerase-positive and negative human cells.[25] Remarkably, this in-frame alternatively spliced isoform is probably the most promising candidate to help us discern TERT functions that require catalytic activity from the ones that do not, since the alternative splicing almost cleanly excises the RT-domain while maintaining an open reading frame and sequence homology with its full-length counterpart. Overexpression of this variant in telomerase-negative cell lines enhanced cell proliferation and LiCl-induced Wnt signaling.[25] However, the response was not as strong as that seen in the full-length hTERT overexpression controls, suggesting that the isoform may merely be carrying out a redundant role of its full-length counterpart.

Lastly, four independent alternative splicing events within the 5′ region of the transcript result in the partial to complete deletion of exon 2 and introduce early PTCs.[25,134] Exon 2 contains portions of the telomerase essential N-terminal domain and RNA-interaction domain, which are lost, along with the catalytic core, in the process of splicing. Translation of the transcripts harboring exon 2 deletions through the wild-type reading frame would render all other potential splicing events downstream of exon 3 defunct due to the PTCs. Of note, the PTC introduced by exon 2 splicing may be bypassed if translation starts at an alternative start site downstream of the wild-type start codon. However, no evidence exists to confirm such a translation pattern to date. Interestingly, Wither *et al.* showed that exon 2-deletion (termed e2) is a common splice event in primates and that the e2 transcripts are highly abundant.[134] In agreement, our preliminary data indicates that the e2 transcripts are present in hESCs and whose amount varies in cells cultured under different oxygen tensions (Betts, unpublished data). Despite the abundance of e2 transcripts, its translational competency is yet to be investigated. Regardless, its naturally high transcript abundance implies that exon 2-deletion may be a non-functional by-product of TERT regulation by alternative splicing, serving as a powerful switch to control telomerase activity and/or non-canonical functions of TERT.

Lack of consistency in methods of comparing isoform transcript abundance and poor antibody availability for TERT and its variants have greatly limited the progress of characterizing alternate splice isoforms of TERT in various species. Nonetheless, newly emerging pieces of evidence indicating the extra-telomeric roles of TERT (Fig. 1) and cell-type specific biases in splicing patterns are supportive of the functional importance of TERT and its variants in pluripotent stem cells. Fig. 1 may be

much more complex with additional isoform specific functions, as well as multiple isoforms modulating each of its current roles.

## Conclusion

Once regarded as a unipartite protein whose sole purpose is to re-lengthen telomeres, TERT is now considered a multi-functional protein, capable of modulating functions beyond telomere maintenance in various cell compartments. It is well accepted that TERT is upregulated in pluripotent stem cells, presumably to support the indefinite cell self-renewal by maintaining telomere lengths. Yet, this understanding lacks appreciation of TERT's possible contribution to pluripotency through its cross talk with the pluripotency network and non-canonical roles. The non-canonical roles of TERT as an active regulator of major signaling pathways, histone modulation, and mitochondrial function have been characterized mostly in a non-PSC context and in different species, making it challenging to gauge the true significance of TERT in PSCs based on our current knowledge of the field. Nonetheless, these functions of TERT, including telomere maintenance, closely relate to a number of highly regulated cell mechanisms of PSCs such as epigenetic and metabolic modulation. Here, we propose ways by which TERT may govern pluripotency and self-renewal in pluripotent stem cells. TERT's canonical and non-canonical functions collectively have a significant impact on pluripotency and self-renewal in pluripotent stem cells.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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