# Dissociation between telomerase activity and expression during mice cortical development due to a DNA-bound telomerase inhibitor

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**Disciplines**
Cell Biology

**Keywords**
Telomere
Brain Development
TERT
DNA-Binding Proteins

**Type of Observation**
Standalone

**Type of Link**
Standard Data

**Submitted** Mar 4, 2016
**Published** May 1, 2016

**Triple Blind Peer Review**
The handling editor, the reviewers, and the authors are all blinded during the review process.

**Full Open Access**
Supported by the Velux Foundation, the University of Zurich, and the EPFL School of Life Sciences.



## Abstract

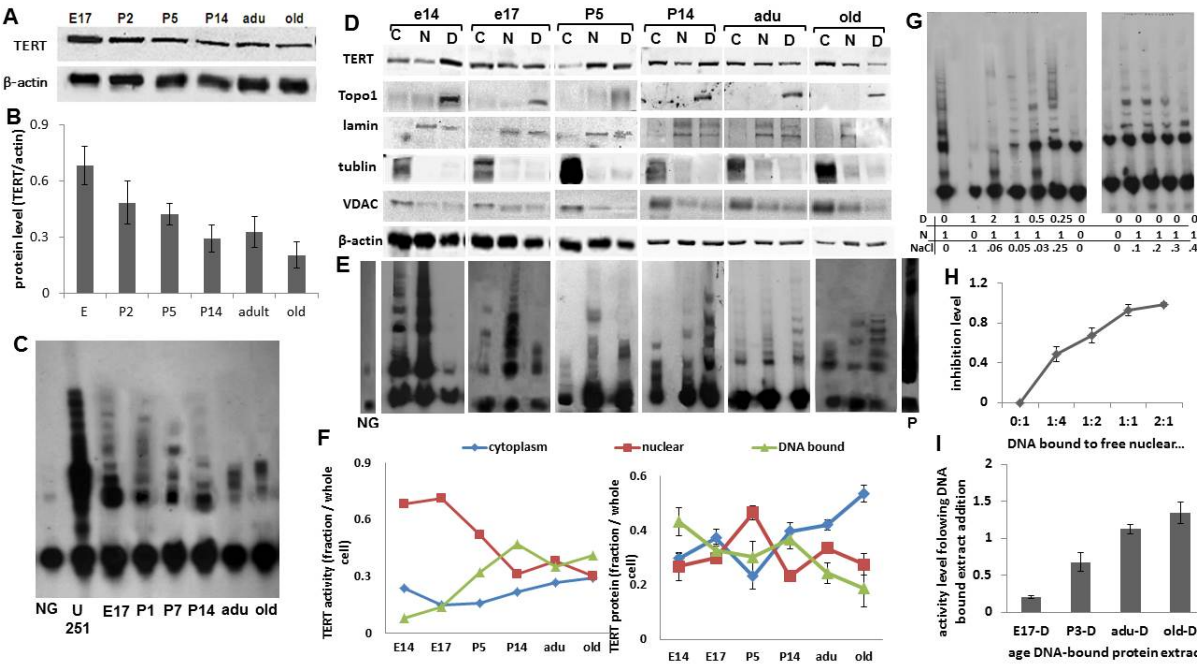
Telomerase, the enzyme that elongate telomeres during DNA replication, is expressed in the neonatal brain and in distinct regions of adult brain. The roles of telomerase in post-mitotic cells such as neurons are mostly unrelated to telomere elongation but rather associated with the promotion of cell survival. Overexpression of TERT, the protein component of telomerase, protects neurons from various insults *in vitro* and *in vivo*. Here we confirm the dissociation between the drastic reduction in telomerase activity during postnatal development and the sustained levels of TERT mRNA and proteins. TERT can interact with the DNA and can be found in the cytoplasm, where it extracts some of its anti-apoptotic function. We measured TERT protein and telomerase activity in cytoplasm, nucleoplasm, and DNA-bound protein fractions during mice cortical development and found a strong dissociation between TERT protein levels and telomerase activity in embryonic DNA-bound protein fraction. Adding the embryonic DNA-bound protein fraction but not an adult DNA-bound protein to telomerase activity reaction significantly inhibited telomerase activity. Thus, we suggest the existence of a yet-unidentified embryo-specific DNA-bound telomerase inhibitor, which may regulate telomerase activity in the developed cortex.

## Introduction

Telomerase is a ribonucleoprotein complex that elongates telomeres; the active enzyme consists of telomerase reverse transcriptase protein (TERT) and an RNA subunit (TERC) [1] [2]. The activity of telomerase *in vivo* requires several additional proteins that are essential for the holoenzyme biogenesis and its trafficking to telomeres [3]. Telomerase is expressed in more than 80% of cancer cell types, in embryonic tissue, and stem cells, but is highly restricted in most mature somatic cells [2]. Telomerase activity was reported in mice embryonic and young neonatal brain, but significantly reduced to undetectable levels at around postnatal day 13. Surprisingly, TERT mRNA and protein are detected in adult and old mice, and recently, it was also found in postmortem human brain [4] [5] [6] [7]. It has been shown that TERT possesses telomere-unrelated functions that can protect neurons from various insults [7] [8]. Thus, TERT may play a role both in brain development and in its response to insults during neurodegenerative disease and acute injuries [9]. Here, we confirm the expression and activity pattern of telomerase in the developing mice cortex and report for the first time that in embryonic cortex TERT is mostly bound to the DNA. Moreover, we observed that this fraction that contains high levels of TERT protein does not support telomerase activity *in vitro* and even abolishes its activity when added to the reaction mixture. These observations suggest the existence of a yet-undefined telomerase inhibitor in the embryonic cortex DNA-bound protein extract.

## Objective

Here we aimed to characterize telomerase expression, activity, and location during mice cortical development.




a

## Figure Legend

**Figure 1. TERT expression and telomerase activity are reduced in the mouse brain cortex with development and aging**.

**(A)** Whole-cell protein extracts were prepared from embryo (E17), postnatal days 2, 5, 14 (P2, P5, P14, respectively), adult (3–5 months), and old (20–22 months) mouse cortex. Protein (25 µg) was analyzed by western blot using anti-TERT antibody. β-Actin was used as a loading control.

**(B)** Quantification of the western blot results (depicted in A) by densitometric analysis using the EZquant software. The results are mean ± SEM of three independent experiments.

**(C)** Telomere elongation activity *in vitro* was measured in the same protein extract as in A by the telomere repeat amplification protocol (TRAP) assay using 1.5 µg of protein extract and 32 PCR cycles. U251 glioblastoma cell line was used as a positive control for telomerase activity. NG – means negative control. This is a representative experiment of n = 3.

**(D)** Cytoplasmic (C), nucleoplasmic (N), and DNA-bound protein (D) fractions were prepared from embryo (E17), postnatal days 2, 5, 14 (P2, P5, P14, respectively), adult (3–5 months), and old (20–22 months) mouse cortex. Protein (25 µg) was analyzed by western blot using anti-TERT antibody, anti-topo1 as a DNA-bound marker, anti-lamin as nuclear marker, anti-tublin as a cytoplasmic marker, and anti-VDAC as a mitochondria marker. β-Actin was used as a loading control. This is a representative experiment of n = 3.

**(E)** Telomere elongation activity *in vitro* was measured in the same protein extract as in D by the telomere repeat amplification protocol (TRAP) assay using 1.5 µg of proteins extract and 32 PCR cycles.

**(F)** Quantification of the western blot and TRAP results (depicted in D and E) by densitometric analysis using the EZquant software. The fraction of TERT in each compartment was calculated by dividing its level in that compartment by the level in all compartments (equation: C or N or D/(C+N+D)).

**(G)** Telomerase activity was assayed by TRAP using a combination of the embryonic nucleoplasm extract and embryonic DNA-bound extract or with the addition of various concentrations of NaCl at the indicated ratio, total protein 1.5 µg, 32 PCR cycles. The final concentrations in the TRAP reaction are 25 fold lower.

**(H)** Quantification of the TRAP results (depicted in G) by densitometric analysis using the EZquant software. The inhibition levels were calculated by dividing the activity level in the combined extract with the activity level of the nuclear extract alone. The x-axis represents the ratio between the nuclear extract and the DNA-bound extract.
**(I)** DNA-bound protein extract derived from mice at the following ages: embryonic day 17 (E17), postnatal day 3 ($P_3$), adult (2–3 months) (adu), and old (20–22 months) were added to embryonic nucleoplasm protein extract in a ratio of 1/1 and telomerase activity was measured by TRAP assay.

**Animals**

CD-1 male mice were used for the investigation of telomerase in the mouse brain. All animal procedures were approved by the animal experimentation ethics committee of our institute.

**Preparation of nuclear and cytoplasmic proteins extracts**

Brains were quickly removed from the skull, washed, and placed in a Ringer solution at 4℃. These were homogenized using a manual homogenizer (pestle B). The homogenates were centrifuged at 500 *g* at 4℃ for 7 min and the pellets were subjected to nuclear and cytoplasmic extractions.

**Cytoplasmic extract**

The cytoplasmic extract was prepared as previously described [12]. Briefly, cells were resuspended in buffer A (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) containing a mixture of protease inhibitors [final concentrations: 2 µg/ml aprotinin, 2 µg/ml antipain, 2 µg/ml leupeptin, 1 µg/ml pepstatin A, 2 µg/ml PMSF (phenylmethylsulfonyl fluoride)], and kept on ice for 15 min. Lysis was performed by passing the solution 10–15 times through a syringe with a 21G needle. The cytosolic fraction was obtained by centrifugation at 9,300 *g* at 4°C for 7 min and the supernatant was collected.

**Nuclear extract**

The pellet from the cytoplasmic preparation was resuspended in CHAPS buffer, containing 10 mM Tris-HCl, 5 mM 2-β-Mercaptoethanol pH 7, 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 0.5% CHAPS (3[(3Cholamidopropyl)dimethylammonio]-propanesulfonic acid), and 10% glycerol, followed by incubation at 4°C for 30 min. The nuclear extract was centrifuged for 30 min at 9,300 *g* at 4°C, and the supernatant was collected.

**DNA-bound protein extract**

The pellet of the nuclear extract was washed with CHAPS twice, each time incubating for 20 min in ice and centrifuged for 20 min at 9,300 *g* in 4°C. Then CHAPS with 1 M NaCl was added and the sample was incubated on ice for 30 min and centrifuged for 20 min at 9,300 *g* in 4°C. The supernatant contained the DNA-bound proteins. Total protein concentration was determined using the BIO-Rad protein assay kit (Bio-Rad Laboratories).

**Separation of proteins by gel electrophoresis and detection**

Protein extracts (as indicated by various experiments) derived from nuclear and cytoplasmic fractions were analyzed by polyacrylamide gel electrophoresis and western blotting as previously described [13], using either anti-hTERT mice IgG hTERT monoclonal antibody (1:1000 1531-1; Epitomics, CA), anti-β-actin (1:7000 Irvine, CA), anti-top1 (1:500 goat IgG sc-26167), anti-lamin B (1:1000 mouse IgG sc-365214) and anti-β-tubulin (1:1000 mouse IgG sc-58886) (Santa Cruz Biotechnology, CA), or anti-VDAC (1:2000 rabit IgG ab34726). The immunocomplexes were detected by enhanced chemiluminescence (Santa Cruz Biotechnology).

**TRAP assay**

Telomerase activity was assessed as described [14]. Briefly, protein extract (at the indicated quantity) was incubated with the reaction mixture (see buffer section) that contains the reaction substrate: TS primer (5′-AATCCGTCGAGCAGAGTT-3') for 45 min at 30℃ followed by PCR assay with [α-P32] dCTP using CX primer (5'-CCCTTACCCTTACCCTTACCCTTA-3') or ACX (5' GCG CGG CTT ACC CTT ACC CTT ACC CTA ACC 3'). Internal standard primers used as a control: IS primer (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3') and ISR primer (5'-ATCGCTTCTCGGCCTTTT-3'). For detection of telomerase products in the brain extracts, 32 PCR cycles were used, and the internal standard primers were diluted to a concentration of $5\times10^{-15}$ M. The PCR products were separated on 12.5% polyacry-

lamide gel, and the radioactive products were detected with phosphoimager (Bio-Rad Laboratories) or by autoradiography using x-ray films.

**Statistical Analysis**

Results are presented as the mean ± standard error of the mean of at least three independent experiments. The statistical significance of the results was obtained using the Student's t-test or one-way ANOVA, using OriginPro7 software. Statistical significance was set at a confidence level of 0.05.

## Results & Discussion

In the cortex, TERT is expressed by only a small fraction of cells, yet it can be detected by western blot, and telomerase activity can be detected by TRAP assays [5]. Mice were sacrificed at different ages (embryonic day 17, postnatal days 2, 5, 14, adult 3–5 months, and old 20–22 months); the cortex was dissected on ice-cold Ringer buffer and whole-cell protein and RNA extract were prepared. The TERT protein levels from the various extracts were analyzed by western blot using anti-TERT and anti-β-actin antibodies. The expression of TERT was significantly reduced during development, then stayed constant between postnatal day 14 and adulthood, and was slightly reduced from adulthood to old-aged mice (Fig. 1A,B). This pattern is similar to the previously reported reduction in TERT mRNA levels during cortex development [10]. Examination of telomerase activity revealed a significant reduction in the activity during mouse cortex development, with only very low levels of activity observed in adult and old mice cortex (Fig. 1C). This is slightly different from the report of Klapper et al. that did not detect any activity in adult mice cortex [4]. It was previously shown that the intracellular localization of TERT changes in response to excitotoxicity or radiation [11]. Here we examined the intracellular localization of TERT during cortex development. Cytoplasmic, nuclear-free, and DNA-bound protein extracts were prepared from the cortex of mice at different ages (embryonic days 14 and 17, postnatal days 5 and 14, adult 2–4 months, and old 22 months) and the TERT protein levels were analyzed by western blot using anti-TERT antibodies (Fig. 1D). The purity of fractions was examined by the presence of specific markers: Topoisomerase I (topo1) for the DNA-bound nuclear fraction, α-lamin for free nuclear fraction, β-tubulin for cytoplasmic fraction, VDAC for mitochondrial fraction, and β-actin was used as a loading control (Fig. 1D). The ability of the same protein extracts to elongate telomeres *in vitro* was measured by the TRAP assay using 1.5 µg protein and 32 PCR cycles (Fig. 1E). The relative quantity and activity of TERT in each protein extract were estimated by dividing its level in the indicated extract by the sum of its level in all the three extracts (the equation is as follows: ((C or N or D)/(C+N+D))). This quantification presented in figure 1F shows that there is a significant increase of TERT level in the cytoplasmic fraction during aging ($n$= 3, $p$ <0.05) and a non-significant decrease of TERT level in the DNA-bound fraction. Telomerase activity in the cytoplasm is slightly reduced with age, while its activity in the DNA-bound extract fraction is remarkably increased in old mice (Fig. 1F). The negative correlation between TERT protein level and telomerase activity in the DNA-bound fractions suggests that telomerase activity is regulated by a yet unknown factor that is present in the DNA-bound fraction and inhibits the activity of telomerase. We examined this hypothesis by a simple experiment in which we mixed different quantities of the embryonic cortex DNA-bound protein extract with the embryonic brain nuclear-free extract that exerts high telomerase activity (Fig. 1G). The result depicted in figure 1G and quantified in figure 1H show that the addition of increasing amounts of embryonic DNA-bound extract exhibits a significant dose-dependent inhibition of telomerase activity in the embryonic-free nuclear extract. In addition, we found that adding NaCl at concentrations lower than 0.3 mM to the TRAP reaction mixture had no effect on the reaction, indicating that the observed inhibition does not appear due to the existence of NaCl in the extract (Fig. 1G, left panel). Finally, we wanted to test if the ability of the DNA-bound fraction to inhibit telomerase activity is specific to the embryonic stage. Therefore, the same aforementioned concentration of the DNA-bound protein extract from the cortex of E17, P3, adult, and old mice was added to embryonic nucleoplasma protein extract and the level of telomerase activity was measured (Fig. 1I). These results show that the telomerase inhibition ability of the DNA-bound protein extract is diminished with age. The identity and the nature of the

inhibitor in the DNA-bound extract is yet unknown.

The current observation shows that TERT protein and activity can be extracted from the DNA fraction in mouse cortex (DNA-bound TERT) as was also previously shown for the mouse cerebellum [11]. In the mouse cortex, the DNA-bound TERT levels reduce during development and aging. In the embryonic cortex although most of the TERT protein is present in the DNA-bound fraction, no telomerase activity can be detected in this fraction. We postulated that the lack of telomerase activity is probably due to the presence of a telomerase inhibitor in this fraction. Indeed adding this fraction to the telomerase activity reaction inhibits telomerase activity. Moreover, this inhibitor is specific to the embryonic fraction, since adding the DNA-bound extract from cortex of postnatal and older mice to an enzymatic active extract has no inhibitory effect. The observation that TERT protein expression is maintained through adulthood while its activity is significantly reduced was reported by previous reports [4] [5] [6] [6]. It will be interesting to examine if this fraction is unique to the brain or can be found in other tissues as well. Finally, while our observation suggests the existence of an embryonic DNA-bound telomerase inhibitor, its nature, identity, and function are not clear. One interesting speculation is that the role of such an inhibitor is to restrict the highly active telomerase in the embryonic stage, avoiding telomere addition in locations other than the end of the chromosomes.

## Conclusions

Surprisingly we found a DNA-bound inhibitor of telomerase activity in the embryonic mice brain, but not in postnatal and adult brain.

## Limitations

The main limitation of the current study is that we did not identify the DNA-bound telomerase inhibitor. Moreover, while we did determine that the inhibitor is specifically present in the embryonic stage, we did not determine if it is specific to the brain.

The current study shows the existence of a DNA-bound telomerase inhibitor in embryonic brain. In our opinion, the most important challenge is to know what its physiological role is. Answering this question requires several steps: identification of the inhibitor by comparing the proteins that interact with telomerase in the embryonic and adult brain. Then knockout experiments should be performed to test its effect on telomere length, intracellular telomerase localization, and possibly brain development.

## Additional Information

### Methods

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## Supplementary Material

Please see https://sciencematters.io/articles/201604000008.

## Ethics Statement

All experiments were done in accordance with the standard guidelines, and animal experiments were approved by the animal experimentation ethics committee.

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