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In vitro models for ectopic expression of telomerase template variants

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Abstract

In most human cancer cells, telomeres are maintained by the telomerase enzyme complex, consisting of a reverse transcriptase (hTERT) associated with a guiding RNA subunit (hTERC) acting as a template for telomeric repeats. The majority of telomeres consist of highly repetitive TTAGGG repeats. Recent studies have also identified non-canonical telomeric repeat (NCTR) variants in human cells, some of which are linked to certain cancer types. This thesis aimed to develop and characterize in vitro cancer cell models overexpressing hTERC with and without mutations in the template region coding for canonical (CTRs) and non-canonical telomeric repeats.

This master thesis project aimed to develop and characterize in vitro cancer cell models overexpressing hTERC with and without mutations in the template region coding for (CTRs) and NCTRs.

Eight cancer cell lines were screened for four suitable candidates for hTERC overexpression experiments using Puromycin cytotoxicity assays and endogenous hTERT/hTERC expression analysis via real-time qPCR (RT-qPCR). Plasmid constructs for hTERC overexpression coding for non-canonical TAAGGG and TGAGGG repeats, and a special variant coding for TCAGGG repeats, were created using site-directed mutagenesis. Subsequently, overexpression of ectopic hTERC coding for CTRs and NCTRs was induced in cancer cell lines HT1080, T98G, Saos2, and U2OS by Lipofection and Retroviral Transduction. Transient and stable total hTERC overexpression, as well as stable integration of the artificial target genes, was evaluated using RT-qPCR. Additionally, telomerase activity of Telomerase-active (TA) overexpression clones (HT1080 and T98G) was assessed using qPCR-TRAP assays. Finally, incorporation of TTAGGG and TCAGGG repeats via Telomerase into artificial telomeric oligomers and genomic DNA was evaluated in selected HT1080 and T98G clones via RT-qPCR and Telo-FISH assays.

Successful transient overexpression of hTERC was shown in HT1080 and T98G clones 48 hours after Lipofection. A significant increase in expression of hTERC was established in all stable overexpression clones of HT1080, T98G, U2OS, and Saos2 after Puromycin selection. Analysis of genomic DNA also suggested stable integration of the target constructs in stable clones. Additionally, overexpression of hTERC induced a significant increase in telomerase activity compared to the untreated parental cell lines in 8 out of 10 established hTERC overexpression clones of the TA cell lines HT1080 and T98G.

Preliminary in vitro analysis indicated elevated TTAGGG and TCAGGG repeat incorporation in artificial telomeric oligomers in the respective HT1080 and T98G clones. No significant increase in NCTR incorporation in genomic DNA was detected in clones with mutant hTERC variants under in situ conditions. However, one HT1080 clone showed significantly increased CTR incorporation after stable wild-type hTERC overexpression.

Overall, hTERC overexpression models were successfully established, with increased telomerase activity in most TA clones. While CTR/NCTR incorporation was limited, these models offer potential for future validation studies of improved qPCR methods for quantifying telomeric repeat variants, offering an alternative to Next Generation Sequencing (NGS).

Zusammenfassung

In den meisten menschlichen Krebszellen werden die Telomere durch den Telomerase-Enzymkomplex aufrechterhalten. Dieser besteht aus einer reversen Transkriptase (hTERT) und einer leitenden RNA-Untereinheit (hTERC) besteht, die als Vorlage für die telomer-Sequenzen dient. Die Mehrheit der Telomere besteht aus hochrepetitiven TTAGGG-Hexameren. Jüngste Studien haben auch so genannte nicht-kanonische telomerische Wiederholungsvarianten (NCTRs) in menschlichen Zellen identifiziert, von denen einige mit bestimmten Krebsarten in Verbindung gebracht wurden. Diese Arbeit zielt darauf ab, in vitro Krebszellmodelle zu entwickeln und zu charakterisieren, die hTERC mit und ohne Mutationen in der Template-Region, die für kanonische (CTRs) und nicht-kanonische telomerische Wiederholungen (NCTRs) kodiert, überexprimieren.

Im Rahmen dieser Masterarbeit wurden acht Krebszelllinien untersucht, um vier geeignete Kandidaten für hTERC-Überexpressionsexperimente auszuwählen, indem Puromycin-Zytotoxizitätstests und endogenen hTERT/hTERC-Expression mittels real-time qPCR (RT-qPCR) durchgeführt wurden. Plasmidkonstrukte für hTERC-Überexpression, die für nicht-kanonische TAAGGG- und TGAGGG-Wiederholungen sowie eine spezielle TCAGGG Variante kodieren, wurden mittels Mutagenese (Site-directed mutagenesis) erstellt. Anschließend wurde die Überexpression von ektopischem hTERC, das für CTRs und NCTRs kodiert, in den Krebszelllinien HT1080, T98G, Saos2 und U2OS mittels Lipofektion und retrovirale Transduktion induziert. Transiente und stabile hTERC-Überexpression sowie die stabile Integration der künstlichen Zielgene wurden mittels RT-qPCR geprüft. Zusätzlich wurde die Telomeraseaktivität von Telomerase-aktiven (TA) Überexpressionsklonen (HT1080 und T98G) mittels qPCR-TRAP-Assays untersucht. Schließlich wurde die Integration von TTAGGG- und TCAGGG-Wiederholungen durch Telomerase in künstliche telomerische Oligomere und in genomische DNA in ausgewählten HT1080- und T98G-Klonen mittels RT-qPCR- und Telo-FISH-Assays bewertet.

Transiente Überexpression von hTERC wurde in HT1080- und T98G-Klonen 48 Stunden nach der Lipofektion nachgewiesen. Ein signifikanter Anstieg der hTERC-Expression wurde in allen stabilen Überexpressionsklonen von HT1080, T98G, U2OS und Saos2 nach Puromycin-Selektion festgestellt.

Die Analyse der genomischen DNA deutete ebenfalls auf eine stabile Integration der Zielkonstrukte in stabilen Klonen hin. Darüber hinaus führte die Überexpression von hTERC zu einer signifikanten Erhöhung der Telomeraseaktivität im Vergleich zu den unbehandelten parental Zelllinien in 8 von 10 etablierten hTERC-Überexpressionsklonen der TA-Zelllinien HT1080 und T98G.

Eine vorläufige in vitro Analyse zeigte eine erhöhte Integration von TTAGGG- und TCAGGG-Hexameren in künstlichen telomerischen Oligomeren bei HT1080- und T98G-Klonen. Kein signifikanter Anstieg an NCTRs in genomischer DNA wurde in Klonen mit mutierten hTERC-Varianten unter in situ Bedingungen festgestellt. Ein HT1080-Klon zeigte jedoch eine signifikant erhöhte CTR-Integration nach stabiler Überexpression von wild type-hTERC.

Zusammenfassung wurden hTERC-Überexpressionsmodelle erfolgreich etabliert, und eine erhöhte Telomeraseaktivität in den meisten TA-Klonen nachgewiesen werden. Obwohl ein Anstieg in CTR/NCTR-Integration nur bedingt nachweisbar war, bieten diese Modelle Potenzial für zukünftige Validierungsstudien zu verbesserten qPCR-Methoden zur Quantifizierung von telomerischen Hexamervarianten. Dies könnte eine Alternative zum aktuellen Gold Standard, dem Next Generation Sequencing (NGS) darstellen.

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1 Aim of the study

The major goal of this master-thesis-project was to create new hTERC-overexpression in vitro models with mutations in the template region in different cancer cell lines using either Telomerase Activity (TA) or Alternative Lengthening of Telomers (ALT) for telomere maintenance. These models may be used as control samples in the validation of new qPCR-assays investigating the role of Non-Canonical Telomere Repeats (NCTRs) in cancer cells.

The project was divided in several consecutive goals as follows:

Analysis of Puromycin resilience and endogenous hTERC/hTERT patterns of eight cancer cell lines.

The first goal was to identify two cell lines with activated telomerase and two cell lines using ALT, to be established as hTERC-overexpression models later on. Therefore, eight cancer cell lines (TA and ALT cell lines) were screened for their puromycin resilience and endogenous expression of both major telomeric subunits hTERC and hTERT using real-time qPCR (RT-qPCR). TA cell lines screened were HT1080 (fibrosarcoma cell line), HeLa (cervical carcinoma cell line), SW480, SW480-LT (adenocarcinoma cell lines) and T98G (glioblastoma cell line). Analysed ALT cell lines were U2OS, Saos2 (osteosarcoma cell line) and YT-BO (glioblastoma cell line).

Establishment of clones expressing mutant and wild type hTERC plasmids in four target cell lines.

In the second phase, five hTERC overexpression plasmids carrying different variants of the hTERC template region were introduced to the four cancer cell lines HT1080, T89G, U2OS and Saos2 by transfection and retroviral infection. These variants carried templates for TTAGGG, TCAGGG, TAAGGG or TGAGGG repeats were introduced (*Figure 1*). The goal was to create clones that transiently, and eventually stably, overexpress hTERC variants introduced by hTERC overexpression plasmids. Transient and stable overexpression of hTERC was evaluated through Real-Time-qPCR (RT-qPCR) following RNA extraction and subsequent cDNA synthesis.

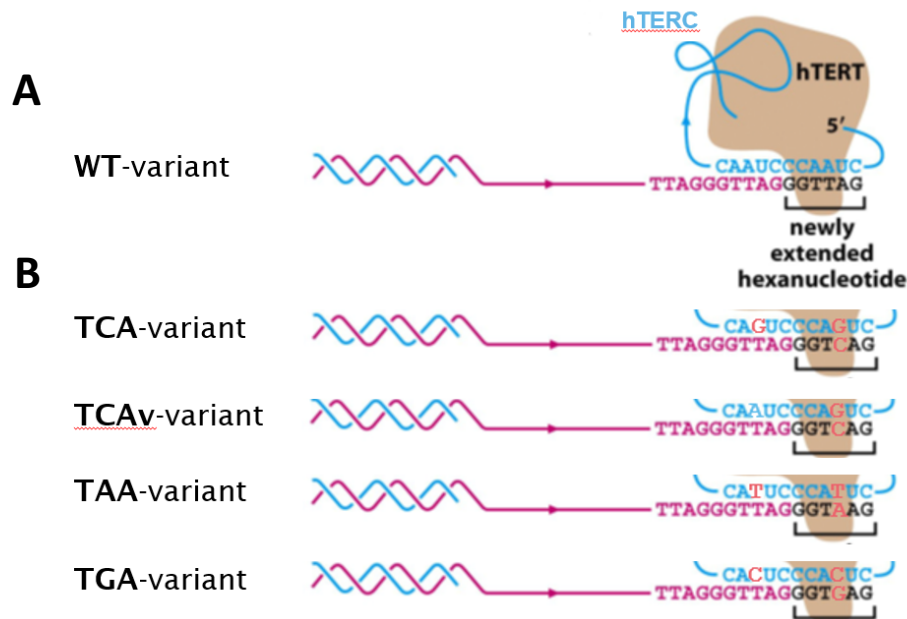


Figure 1 – (A) Schematic figure of the telomerase complex bound to the 3' end of the telomeric regions depicting the catalytic subunit hTERT in brown and the coding RNA subunit hTERC in blue. The 11 nucleotides of hTERC (in blue) responsible for binding to the correct site (CAAUC) on the telomeric region and coding for the synthesis of the newly extended hexanucleotide (CCAAUC) are shown here. **(B)** Modified variants of the artificial hTERC are listed below the WT-variant, showing both nucleotide substitutions (red) introduced on the template region by site directed mutagenesis, with one nucleotide change on the binding region and one on the coding region, as done in previous studies ¹. A second version of the TCA-variant called TCAv was created with only one nucleotide substitution on the coding region, leaving the binding region unmodified. This figure was adapted from Figure 10.24 – The Biology of Cancer ².

Characterization of stable clones overexpressing hTERC variants regarding hTERC gene copy numbers, Telomerase activity and telomeric repeat patterns.

In the final phase, characteristics of up to 19 clone bulks stably overexpressing various hTERC variants were evaluated. Gene copy numbers of the introduced target genes were analysed using RT-qPCR to confirm successful integration. To evaluate if overexpression of hTERC subunits influences activity of the whole telomeric protein complex, telomerase activity was measured using the RT-qPCR telomeric repeat amplification protocol (RT-qPCR-TRAP). Additionally, in vitro functionality of ectopic hTERC in complex with endogenous hTERT was tested using an experimental combinatorial TRAP-qPCR assay from Glukhov et al. 2021 ³. Finally, to determine whether overexpression of hTERC variants results in increased incorporation of the respective telomeric repeat variants into telomeres, the telomeric sequences of selected TA clones were analysed using RT-qPCR and telomere-specific

fluorescence in situ hybridization (telo-FISH) assays, quantifying canonical and non-canonical telomeric repeats.

2 Introduction

Telomers are nucleoprotein complexes located at the end of linear chromosomes consisting of highly repetitive DNA repeats associated with specialised proteins, so called shelterins ⁴. Their structure primarily consists of TTAGGG hexameric-repeats ranging from 2-50 kilobases containing approximately 300-8000 repeats in length ⁵. Their total telomere lengths can vary between individuals of one species as well as between individual chromosomes of a single cell. Telomers of human cells are approximately 10 to 15 kilobases long ^{6,7}.

The most commonly known function of telomers is to prevent the coding genome from DNA-damage during replication and to protect the chromosomal ends from misguided DNA-damage responses ^{4,8}.

After each replication cycle 50-200 bp of a human chromosome are lost on the 5'-end of the lagging strand due to incomplete DNA syntheses. DNA-Polymerase-I usually fills the gaps between an Okazaki-fragment and the next upstream RNA-Primer. However, the very last terminal fragment of the lagging strand cannot be synthesised due to the lack of another upstream RNA-primer, resulting in the loss of those non-replicated bases. This phenomenon is called the "End-Replication-Problem" ⁹. Telomers serve as buffer sequences, to keep functional regions from getting shortened during the replication process. Furthermore, formation of a large terminal loop structure at the 3'end of the telomers protects chromosomal ends from chromosomal fusions by DNA-damage response pathways ^{4,10}. This so-called T-loop structure is the result of a single stranded 3' overhang forming a loop invading the double stranded region upstream by creating a pocket through formation of a D-loop, facilitated by the shelterin complex (*Figure 2*).

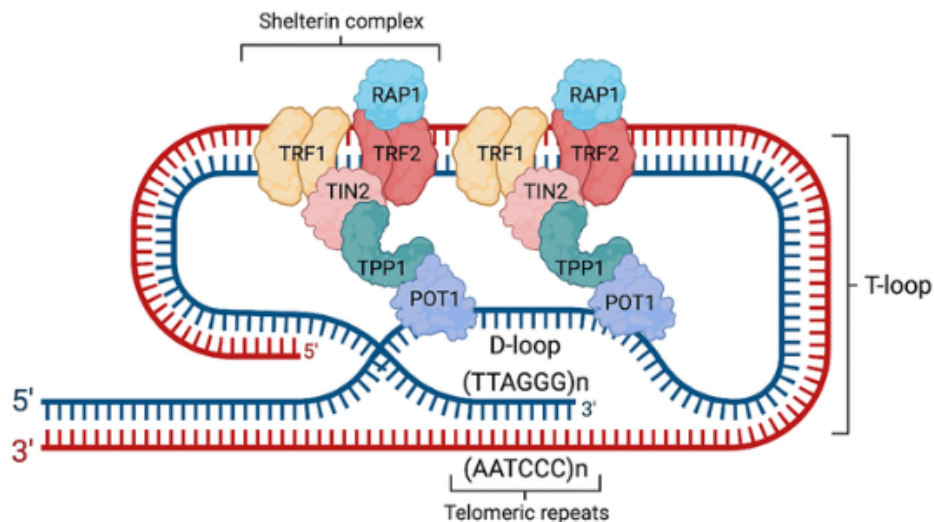


Figure 2 – T-loop structure at the end of the telomeric region. Single-stranded 3' overhang invades double-stranded region upstream, hybridising with complementary region in the D-loop. The shelterin protein complex facilitates and stabilizes both loop structures ¹¹.

When the telomere length of a cell reaches a certain limit of replication cycles, the so-called Hayflick-limit ¹², the cell goes into senescence, preventing further DNA-damage by replication. This crucial mechanism protects the coding genome from DNA-damage and acts tumour suppressive, stopping cells from reaching a state of uncontrolled proliferation eventually. Nevertheless, cancer-cells are able to make use of certain Telomere Maintenance Mechanisms (TMMs), enabling them to avoid telomere shortening ¹³. By activation of TMMs cancer cells are able to lengthen their telomers by adding new telomeric repeats to the telomers.

The two major TMMs are activation of the telomerase complex (TA) and alternative lengthening of telomers mechanisms (ALT). The telomerase complex, first described by Carol W. Greider and Elizabeth H. Blackburn in 1985 ¹⁴, is a Ribonucleoprotein-complex (RNP) consisting of an enzymatic subunit *telomerase reverse transcriptase* (TERT) and an RNA subunit *telomerase RNA component* (TERC). The human TERC (hTERC) is a 451 bases long non-coding RNA responsible for binding to the 3' end of telomers and acting as template for the synthesis of new telomeric repeats. hTERC contains an 11 bases long template region 3'-CAAUCCCAAUC-5' with the first 5 bases (underscored) responsible for guiding the telomerase complex to the correct target sequence, and the last 6 bases acting as template for the synthesis of new repeat extensions ^{15,16}. Acting as primer, hTERC enables the human TERT

(hTERT) to synthesize new telomeric repeats. Human telomerase is only active in stem cells and germ cells, and silenced in somatic cells. However, most cancer types are able to reactivate the telomerase during tumour progression, allowing them to maintain stable telomere lengths despite rapid proliferation¹³. Thereby unlocking limitless replicative potential, which has been described as one of the hallmarks of cancer development^{2,17}.

Cancer cells using ALT on the other hand, take advantage of DNA replication mechanisms, using the highly repetitive sequences of their existing telomers to extend them by homologous recombination^{18,19}.

Either ALT or TA can be found active in almost all known human tumour types. Telomerase-dependent telomere maintenance (TA) is prevalent in 80-90% of all tumours^{13,20}, while ALT-mechanisms are active in only of known tumour types^{21,22}.

Sometimes elongation of telomeric sequences by TMMs leads to incorporation of altered telomeric repeats into the cell's genome. Those non-canonical-telomere repeats (NCTRs) are similar to the well-known canonical TTAGGG telomeric repeat, most often distinguishing themselves by one or two nucleotide substitutions on the first 3 positions of the hexameric repeat²³. NCTRs are known to be present in different variations and numbers in human chromosome ends, especially on the proximal regions of the telomeric sequences. Common variant-repeats for example are TCAGGG, TGAGGG, TAAGGG and TTGGGG^{24,25}, varying in number between individual chromosomes as well as tissues.

It is known that NCTRs are also prevalent in all human telomers. However, telomers of human cancer cells often show structural differences in their NCTR patterns and telomere lengths, depending on whether they are maintained by TA or ALT²³.

TA cells frequently produce variants with exchanges on position 1 and 3 of the hexameric repeats, including the variations mentioned above. This incorporation of repeat variants is hypothesized to be caused by the error-prone reverse transcriptase activity of the hTERT subunit mis-incorporating single nucleotides along the RNA-template²³. Cells using TA generally show more homogenous telomere lengths on their chromosomes with comparably short total lengths. ALT-cells on the other hand, have longer and more heterogeneous telomere lengths, and express characteristic extrachromosomal telomeric DNAs^{26,27}. Furthermore, it has been shown, that NCTR-patterns of ALT cells differ from TA cells, by an

increased amount of TCA-variant repeats incorporated into their telomers, as depicted in *Figure 3*¹.

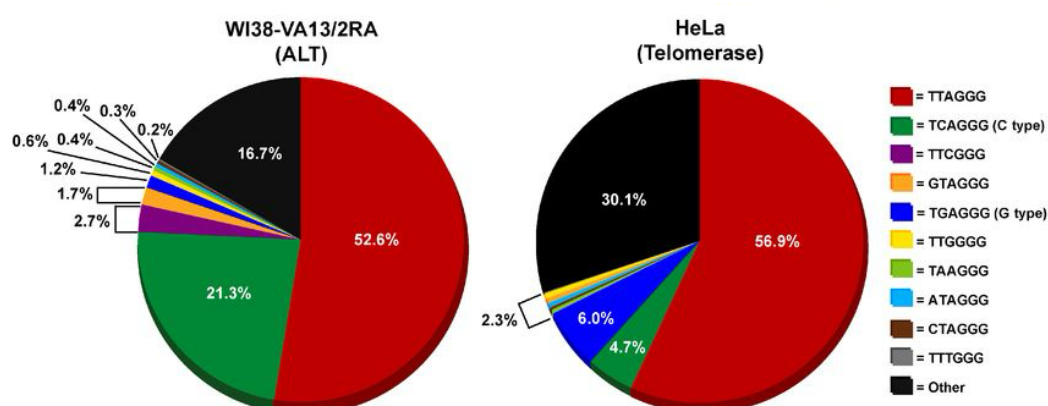


Figure 3 – Next- generation-sequencing data comparing telomeric repeats variants of immortalized WI38-VA12/2RA cells using ALT to HeLa cancer cells using TA. WI38-VA12/2RA cells show a strong increase of TCAGGG repeats compared to HeLa cells¹.

As established, there are characteristic differences in telomere repeat patterns and telomere lengths between cells using different TMMs. Yet, it is not clear how these characteristic differences in telomeric structure can be linked to different cancer types, especially regarding pathogenicity and cancer progression. Using analysis of telomere structures of cancer tissue as a tool to link their structure to certain properties of cancer types, is the first step to establish new quick and efficient methods diagnosis of tumour-linked diseases and telopathies^{28,29}. However, due to the highly repetitive patterns inherent to telomers, comprehensive quantitative analysis of their structures has been difficult to accomplish. The current gold standard for quantitative analysis of telomers is Next-Generation-Sequencing (NGS). NGS has proven to be a useful method to gain a better insight into telomeric structures of cancer cells. However, it is still a complex and expensive method generating huge datasets requiring further bioinformatical analysis to make use of the generated data. Although improved qPCR-assays like Cawthon's monochrome multiplex PCR provide a simpler and more efficient alternative to quantify the canonical telomeric repeats, no such assays have yet been developed for NCTRs³⁰. To find a more efficient, simpler, and cheaper alternative to NGS, validation of qPCR assays quantifying both canonical and non-canonical telomeric repeats is required.

To verify the efficiency in detection of certain non-canonical repeats, the use of control samples expressing characteristic NCTR patterns is a major checkpoint in establishing such qPCR assays. Possible control samples for example may be collected from cancer cell lines used in prior studies evaluating telomeric repeat patterns, or from cancer cell lines with artificially increased incorporation of NCTRs by overexpression ectopically introduced hTERC-template variants. Similar artificial expression systems for wild-type and mutant hTERC genes have already been described for in vitro cell lines in several studies ^{1,31,32}.

3 Material and Methods

3.1 Cell culture

All cell lines except YTBO and SW480-LT were originally obtained from ATCC (The American Type Culture Collection). ATCC numbers of ATCC cell lines are described brackets below. SW480 (CCL-228) is a colorectal adenocarcinoma cell line. SW480-LT is a sub cell line of the SW480 with longer telomeres (LT). Both cell lines were obtained from Robert Mader (General Hospital Vienna) and Brigitte Marian (Medical University Vienna, Austria), respectively³³. Cell line YTBO was initially established from a patient diagnosed with glioblastoma multiforme at the Medical University of Vienna (MUW, Austria)³⁴. Osteosarcoma cell lines U2OS (HTB-96) and Saos2 (HTB-85) were kindly provided by Ingrid Walter from Veterinary University Vienna. YTBO, cervical carcinoma cell line HeLa (CCL-2), fibrosarcoma cell line HT1080 (CCL-121) and glioblastoma cell line T98G (CRL-1690) were provided from cell culture core facility of the Center for Cancer Research of the Medical University Vienna, as well as fibroblast cell line WI38-VA13-2RA (CCL-75.1), epithelial kidney cell line HEK293 (CRL-1573) and myeloid leukaemia cell line K562 (CCL-243) (*Table 1*).

HELA, SW480, SW480-LT, YTBO and K562 were maintained in RPMI-1640 Medium (R). DMEM medium was used for culturing HT1080, U2OS, Saos2, HEK293 and WI38-VA13-2RA. T98G was maintained in MNP medium (M). Additionally, each growth medium was supplemented with 10% Fetal Calf Serum (FCS), 1% Penicillin-Streptomycin (P/S), 1% Non Essential Amino Acids (NEAA), and 1nM Sodium Pyruvate (NaP). All cell lines were incubated in an incubator set to 37°C and 5% CO₂.

Clone bulks of cells introduced to overexpression plasmids were selected for three to twelve weeks using Puromycin added to the respective media. Selection of lipofected and retro-viral infected clone bulks started 3 days after transfection/infection. Depending on their proliferative activity, clone bulks were split regularly once or twice a week. Clone bulks of HT1080, U2OS, HeLa, SW480, SW480-LT, K563 and HEK293 were split 1:10 twice a week, while clone bulks of T98G and Saos2 and YTBO were split 1:5 only once a week. Cell samples for DNA-, RNA- and protein-analysis were taken from the cultures at 70%-90% estimated confluence, representing one end point each. Cell counts were determined with an automated cell counter (Casy TTC, OLS).

Table 1 - Overview of cell lines and their used TMM based on literature

Cell line	Origin/Type	TMM
HEK293	Epithelial kidney cells	TA ³⁵
HELA	Cervical Carcinoma	TA ³⁵
HT1080	Fibrosarcoma	TA ³⁶
K562	Chronic Myeloid Leukemia	TA ³⁷
Saos2	Osteosarcoma	ALT ³⁸
SW480	Colorectal Adenocarcinoma	TA ³³
SW480-LT	Colorectal Adenocarcinoma	TA ³³
T98G	Glioblastoma Multiforme	TA ³⁹
U2OS	Osteosarcoma	ALT ³⁸
WI38-VA13-2RA	Fibroblast	ALT ¹
YTBO	Glioblastoma Multiforme	ALT ⁴⁰

3.2 Kill curves and SRB Assays

For each cell line (Hela, HT1080, Saos2, SW480, SW480-LT, T98G, U2OS and YTBO), a half 96-well plate was seeded with 2000 cells in 100µL medium per well and incubated at 37°C overnight. On the next day, 100µL medium plus Puromycin per well were added, so that the total volume of 200µL per well had the target concentration followed by incubation at 37°C for 5 days. Target concentrations of the preliminary experiment were 0 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL, 5 µg/mL and 10µg/mL Puromycin in per well. Target concentrations of the final experiment were 0 µg/mL, 0.1 µg/mL, 0.2 µg/mL, 0.3 µg/mL, 0.4 µg/mL, 0.5 µg/mL, 1 µg/mL and 5µg/mL Puromycin in media. Each concentration was distributed over 8 wells per cell line. After 5 days incubation was stopped, and SRB-Assays were used to determine the remaining relative viable cell numbers of each cell-line.

The SRB-Assay was performed as described by Erdem et al in 2017.⁴¹ The supernatant of 96-well plate cultures from kill curves was aspirated with waterjet-pump, 80µL 10% Trichloroacetic acid (TCA) was pipetted into each well and incubated at 4°C for 1 hour. After incubation, TCA-supernatant was discarded and each plate was washed three times carefully with purified water. After removal of residual liquid, 80µL of 0.4% Sulphorhodamine B-Dilution (SRB – diluted in 1% Acetic acid) was added to each well and plates were incubated for 15 min. at room temperature. SRB-dilution was removed and plates were washed three times with 1% Acetic acid to remove remaining SRB-dilution. Then, 200µL 10mM TRIS-HCl was added to each well and plates were shaken for 15 min. to extract SRB-dye from cultures. Absorbance was measured in a TECAN Infinite 200Pro plate reader at a wavelength of 570nm.

3.3 Site directed Mutagenesis

Plasmid variants coding for expression of ectopic hTERT mutants TCA-v, TAA-, and TGA- were created using the NEB Q5-Site-Directed-Mutagenesis Kit (E0554S) and the pBABE-Puro-U3-hTERT-WT-500^{1,42} plasmid as template plasmid. The mutagenesis primers used were created using the *NEBase Changer Online Tool* (Table 2).

Table 2 - Mutagenesis primers created and used.

Mutagenesis Primer	
TCA-v forward	5'- CTTACTGAGAAGGGCGTAGGC -3'
TCA-v reverse	5'- GGTAAGACAAAAAATGGCCACCAC - 3'
TAA forward	5'- CTTACTGAGAAGGGCGTAGGC -3'
TAA reverse	5'- GGTAAGACAAAAAATGGCCACCAC - 3'
TGA forward	5'- CTCAGTGAGAAGGGCGTAGGC -3'
TGA reverse	5'- GGTGAGACAAAAAATGGCCACCAC - 3'

Primer-mixes for both Mutation and Control reaction were prepared as follows. The Mutation primer-mixes were created by mixing 2µl of the respective forward primer and 2µl of the

respective reverse primer with 16µl ddH₂O to a total volume of 20µl primer-mix with a concentration of 10µM. For the control reaction, the kit SDM Primer Mix was used per manufacturer's instructions. The target plasmid dilution of pBABE-Puro-U3-hTERC-WT-500^{1,42} was prepared with 0.1x TE-buffer to an end concentration of 5ng/µl. The final reaction volumes were prepared as stated below in thin-walled PCR tubes (*Table 3* and *Table 4*).

Table 3 - *Mutagenesis reaction reagents and volumes*

MUTAGENESIS REACTION	25µl REACTION	FINAL CONCENTRATION
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
Primer Mix (10µM)	1.25 µl	0.5 µM
Template DNA (5 ng/µl)	1 µl	5 ng
Nuclease-free Water	10.25 µl	

Table 4 - Control reaction reagents and volumes

CONTROL REACTION	25µl REACTION	FINAL CONCENTRATION
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µl	1X
Control SDM Primer Mix	1.25 µl	0,5 µM
Control SDM Plasmid	2 µl	10 ng
Nuclease-free Water	9.25 µl	

After all reagents were combined in the respective reaction mixes and thoroughly mixed, the reaction volumes were incubated in a BIORAD T100 thermal cycler using the program depicted in *Table 5*.

Table 5 – Thermal cycler program

STEP	TEMPERATURE	TIME
Initial Denaturation	98°C	30 sec.
25 Cycles	98°C	10 sec.
	64°C	20 secs
	72°C	1 min. 45 sec.
Final Extension	72°C	2 min.
Hold	4°C	

The linear PCR products were then adjoined to fully functional circular plasmids using the KLD-treatment from the kit by mixing the PCR-products with the KLD-Mix (*Table 6*) and incubating the mix for 5 min. at room temperature (RT).

Table 6 - KLD reaction reagents and volumes

KLD REACTION	10µl REACTION	FINAL CONCENTRATION
PCR-Product	1 µl	
KLD-Reaction Buffer	5 µl	1X
KLD-Enzyme Mix	1 µl	1X
Nuclease-free Water	3 µl	

The final hTERC-variant plasmids and Control-Plasmids were transformed into NEB 5-α Competent E.coli with the NEB Q5-Site-Directed-Mutagenesis Kit (E0554S) (see *Chapter 3.3* for details). After confirmation of successful completion of the mutagenesis step using the control plasmids, the new hTERC-plasmids were isolated using Plasmid MiniPrep from single

clones (see *Chapter 3.5* for details). The correct target mutations were tested using Sanger-Sequencing (see *Chapter 3.7* for details). After confirmation of the successful introduction of the target mutations, the plasmids were amplified again by Plasmid MaxiPrep (see *Chapter 3.6* for details).

3.4 Transformation into NEB 5- α Competent E.coli

One tube of NEB 5- α Competent E.coli was mixed with 5 μ l KLD-treated plasmids from the NEB Q5-Site-Directed-Mutagenesis experiments for each plasmid variant. As transformation control, 1 μ l of pUC-control plasmids (50pg/ μ l) was added to another NEB 5- α Competent E.coli tube. The mixtures were put on ice for 30min., followed by heat shock at 43°C for 30 sec. and subsequential incubation on ice for another 5 min. Then each mixture was mixed with 950 μ l SOC (delivered with NEB-mutagenesis-Kit) at room temperature, followed by incubation for 60 min. at 37°C and 210 rpm on a thermos-shaker. Finally, each culture was spread on Ampicillin-selection plates in a 1:40 dilution as well as undiluted with a total volume of 50 μ l per plate. Control cultures for Site-Directed-Mutagenesis were plated separately on LB-Amp-X-gal-IPTG plates (100 μ l/mL Ampicillin + 40 μ g/mL X-gal + 50 μ g/mL IPTG in LB-Agar). All plates were incubated over night at 37°C.

3.5 Plasmid Mini Prep

The plasmids *pBABE-puro-U3-hTR-wt-500*^{1,42}, *pBABE-puro-U3-hTR-TCA-500*^{1,42}, *pBABE-puro-U3-hTR-TCA-v-500*, *pBABE-puro-U3-hTR-TAA-500*, *pBABE-puro-U3-hTR-TGA-500* and *pBABE-puro-GFP* (Table 7) were amplified in competent E. coli cultures. For each plasmid, a single E.coli clone was selected by dilution plating and cultivated in 5mL Lysogeny Broth with Ampicillin (0.1%) at 37° overnight. A total of 4.5mL of each overnight culture was centrifuged for 3 min. at 4°C and 10.000g. The supernatants were discarded, E.coli-pellets were resuspended in 110 μ l STETL-buffer (8% Saccharose, 0.5% TritonX-100, 50mM Tris pH 8, 50mM EDTA, Lysozyme 1X) and incubated for 60 sec. at 95°C, followed by a centrifugation step for 10 min. at 4°C and 10.000g. Debris-pellets were discarded, 2 μ l RNase A were added to supernatant and mixed 1:1 with isopropanol to precipitate the DNA. After centrifugation for 15 min. at 4°C and 10.000g, supernatant was discarded and pellets washed with 150 μ l ice

cold 70% Ethanol, and left to dry for 10 min. Finally, dried pellets containing the targets plasmids were resuspended in 30µl 1xTE, DNA concentration was measured in a NanoDrop spectrophotometer. Successful introduction of the target mutations.

Table 7 – Overexpression plasmids used in this study

NAME	ORIGIN	PLASMID MAP
<i>pBABE-puro-U3-hTR-WT-500</i>	Fu et al. 2003 ⁴² / Conomos et al. 2012 ¹	<i>Figure S 2A</i>
<i>pBABE-puro-U3-hTR-TCA-500</i>	Fu et al. 2003 ⁴² / Conomos et al. 2012 ¹	<i>Figure S 2B</i>
<i>pBABE-puro-U3-hTR-TCA-v-500</i>	Created from <i>pBABE-puro-U3-hTR-WT-500</i> using site-directed mutagenesis (Chapter 3.3)	<i>Figure S 2C</i>
<i>pBABE-puro-U3-hTR-TAA-500</i>		<i>Figure S 2D</i>
<i>pBABE-puro-U3-hTR-TGA-500</i>		<i>Figure S 2E</i>
<i>pBABE-puro-EGFP</i>	Donation from Assoc. Prof. Priv.-Doz. Mag. Dr. Michael Grusch	<i>Figure S 3</i>

3.6 Plasmid Maxi Prep

All the plasmids (*Table 7*) were amplified in competent *E. coli* cultures. For each plasmid, a single *E. coli* clone was selected by dilution plating and cultivated in 225mL Lysogeny Broth with Ampicillin (0.1%) at 37°C for 24 hours. Then cultures were centrifuged at 6000rcf and 4°C to separate cells from media. The cell pellets were stored at -20°C for further use.

For the plasmid DNA purification from the *E. coli* amplification cultures, the EndoFree Plasmid QUIAGEN Maxi Kit (Cat. No. / ID: 12362) was used. The working steps were followed exactly as described in the EndoFree Plasmid Purification Handbook. DNA-concentrations and purity were measured using NanoDrop measuring absorbance at 260nm.

3.7 Sanger-Sequencing

The correct target sequences marking the introduced hTERC-mutations on the pBABE-Puro-U3-hTR-500 plasmid-variants were verified by sanger-sequencing performed by *Microsynth AG*. The target-regions including the PuroR-gene, the ectopic hTERC-gene and the 3'exohTERC region were sequenced using three sequencing primers (*Table 8*). The final sequences were aligned using the *Clone Manager 9* software and the template region with introduced mutations marked in *Figure 1* and *Figure S 2*.

Table 8 - Sequencing Primers

Sequencing Primer 1	5'-AGCTGTTCCATCTGTTCTGAC-3'
Sequencing Primer 2	5'-ATTCGCCCTATAGTGAGTCG-3'
Sequencing Primer 3	5'-TGCAAGAACTCTTCCTCACG-3'

3.8 Lipofection

Cell lines Saos2 and T98G were transfected with plasmids coding for WT and TCA hTERC variants. Cell lines HT1080 and T98G cell-lines were transfected with plasmids coding for WT, TCA, TCAv, TAA and TGA hTERC variants (*Table 7*). Additionally, all four cell lines were transfected with the pBABE-Puro-EFPG plasmid. As negative control, one well of each cell line was incubated with serum free media instead of transfection-mix. Cell lines were seeded with 3×10^5 cells/well on 6-well plates and incubated for 24 hours at 37°C prior transfection. Transfection master mixes were made for each plasmid and incubated at room temperature for 20 minutes. Each mastermix contained 1µg plasmid DNA in 250µl serum free media and 5µl BIORAD-TransFectin™ reagent in 250µl serum free media combined to a total volume of 500µl per well. After incubation for 20 min., growth media of each prepared culture was removed and 500µl transfection-mix was added dropwise to each well. Transfected cultures were then incubated for 4 hours at 37°C. After incubation, media was exchanged with the appropriate growth media to allow proliferation of transfected cells. Each cell line was transfected in duplicates. 48 hours after transfection, one duplicate of each clone per cell line was harvested for analysis of transient hTERC expression. The rest of the cells were incubated for another 24 hours, before selection of stable clones with 1µg/mL puromycin was added to the growth medium. Transfections were performed as one independent experiment.

3.9 Retrovirus production and retroviral infection

Prior to infection of the target cells, active retroviruses carrying the target genes coded on the overexpression plasmids were assembled and amplified in transfected HEK293 cells using a standard protocol⁴³. For each plasmid variant, a T25 cell culture flask was seeded with 1×10^6 HEK293 cells/flask. On the next day, all target plasmids (*Table 7*) were co-transfected into the

prepared HEK293-cultures together with the packaging plasmids *pgag-pol-gpt* and *pVSV-G* using CaCl_2 -transfection.

For each CaCl_2 transfection reaction mix, 0.5mL of 2X HBS Buffer was aliquoted in a 15mL Falcon tube and kept at room temperature. A plasmid mix containing 3.75 μg *pgag-pol-gpt* plasmid (A169) and 1.25 μg *pVSV-G* plasmid (A168) were mixed with nuclease-free H_2O (Promega) to a total volume of 250 μL per transfection reaction.

Subsequently 250 μL of the plasmid mix were mixed with 61 μL 2M CaCl_2 solution, 5 μg pBABEpuro-target plasmid and nuclease-free H_2O to a total volume of 500 μL . Using an empty 5mL glass pipette, air was injected into the falcon tube containing 0.5mL of 2XHBS, while simultaneously adding the plasmid- CaCl_2 mix and incubating at room temperature for another 15-20 minutes. After incubation, the mixture was added to the HEK293 cells in a T25 flask with fresh 2.5mL DMEM medium with 5% FCS. Following incubation at 37°C for 3-5 hours, medium of the transfected cells was changed to 5.5mL of DMEM medium with 10% FCS and 1% P/S.

After three days of incubation, the supernatant containing the active retroviruses coding for the respective target genes were harvested from the transfected HEK293 cells by sterile filtration and stored at -20°C.

For the infections, the target cell lines HT1080, T98G, U2OS and Saos2 were seeded on 24-well plates with 5×10^4 cells/well. HT1080 and U2OS were infected with all five hTERC-variant viruses assembled, while T98G and Saos2 were only infected with the WT- and TCA-variants (*Table 7*). Infections of HT1080 and U202 with WT- and TCA- variants were performed in two repetitions. All other infections were performed in one independent experiment. As positive controls, each cell line was infected with a GFP-expression virus. As negative control, one well per cell line was incubated with medium only. Each culture was incubated over night with 0.5mL of the respective retroviral suspension spiked with 5 μL Polybrene. Then, the infected cells including the negative control were incubated for another 48 hours with standard growth media, before selection of stable clones with media containing puromycin. Retroviral infections of

Establishment of the retrovirus carrying the target sequences as well as retroviral infections of target cell lines was performed with the help of laboratory technician Doris Mejri in a special laboratory for biosafety level 2 as required.

3.10 DNA Isolation and quantification

Cell samples for DNA isolation were collected from clone cultures at 70-90% confluence and centrifuged at room temperature for 5 min. at 1000 rcf. Pellets were washed with PBS, centrifuged at 4°C for 10 min. at 800 rcf, and stored at -80°C until further processing. Genomic DNA from the cell pellets was isolated using the Puregene Cell Kit (Cat. No. / ID: 158043). Adapted from the Puregene Cell Kit manual DNA Purification from Cultured Cells the pellets were processed as follows. Pellets were kept on dry ice, incubated for 1 min. at 37°C slowly shaking and resuspended with 400µl Cell Lysis Solution. Lysate were then incubated at room temperature overnight. After incubation, 2µl RNase A Solution was added to each lysate, followed by incubation for 15 min. at 37°C. After cool-down for 60 sec. on ice, lysates were mix with 133µl Protein Precipitation Solution, vortexed for 20 sec. at 1800 rpm and centrifuged for 1 min. at room temperature and 16000 g. Lysates were then incubated on ice for 5 min. a centrifuged again for 1 min. at room temperature and 16000 g. Supernatant was transferred into a new tube with 300µl Isopropanol, carefully mixed and centrifuged again for 1 min. at room temperature and 16000 g. After DNA precipitated, supernatant was discarded, and the DNA-pellets were washed with 300µl 70% Ethanol followed by a final centrifugation step for 1 min. at room temperature and 16000 g. Supernatant was discarded again and DNA-pellets were left to dry for 10 min and resuspended in 50µl DNA Hydration Solution. The DNA solutions were incubated for 60 min. at 65°C and left shaking over night at room temperature at 200 rpm.

Concentrations of the final DNA-extracts were measured with Nano-Drop at 260nm and *iQuant High Sensitivity dsDNA Quantitation Kit (N013)* according to the manufacturers protocol. Until further analysis samples were stored at -20°C.

3.11 RNA Isolation and Quantification

TRIZOL Reagent (Invitrogen, USA) was used to purify RNA from cell pellets. Cell pellets were collected from cell cultures at 70-90% confluence, and centrifuged at RT for 5 min. at 1000rcf. Pellets were washed with PBS, centrifuged at 4°C for 10 min. at 800rcf and stored at -80°C until further processing.

Frozen cell pellets were kept on ice and resuspended in 250 μ L TRIZOL reagent each. After incubation for 5 min. at RT, 100 μ L Chloroform were added to each lysate to induce phase separation, followed by vortexing for 15 sec. and incubation at RT for 2-3 min. Lysates were then centrifuged at 4°C for 15 min. at 12000rcf. The upper hydrophilic phase was separated from two lower phases, mixed with 250 μ L Isopropanol and incubated at RT for 10 min. The mixture was then centrifuged again at 4°C for 10 min. at 12000rcf. The supernatant was removed leaving mostly visible RNA-pellets in the tube. RNA-pellets were washed with 500 μ L 75% Ethanol and centrifuged 4°C for 5 min. at 7500rcf. Supernatant was aspirated and pellets were left to dry at RT for 10-30 min. Finally, dry RNA-pellets were resuspended in 30 μ L RNase-free double-distilled water with 1% heat resistant RNasin Inhibitor (40U/ μ L) and stored on ice.

RNA-concentrations and purity were measured by NanoDrop measuring absorbance at 260 and 280nm. RNA-samples were stored at -20°C until further use.

3.12 CHAPS protein extraction

Cell pellets were collected from cell cultures at 70-90% confluence and centrifuged at RT for 5 min. at 1000 rcf. Pellets were washed with PBS, centrifuged at 4°C for 10 min. at 800 rcf, shock-frozen in liquid nitrogen and stored at -80°C until further processing. For protein extraction, frozen pellets were kept on dry ice until and resuspended with 200 μ L 1x CHAPS-Buffer (10 mM Tris-Cl pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM Benzamidine, 5 mM B-Mercaptoethanol, 0.5% CHAPS, 10% Glycerol). CHAPS-Buffer used for protein-extraction was spiked with RNase-, Protease- and Phosphatase-inhibitors to maintain the functionality of the isolated proteins as best as possible. The lysates were incubated on ice for 30 min. and centrifuged for 20 min. at 4°C and 12000 g. The supernatant containing the isolated proteins was transferred into a new tube, shock frozen on dry ice and stored at -80°C.

Protein concentration of each sample was measured using the Bradford Assay. Each protein lysate was diluted 1:4 mixing 8 μ L lysate and 24 μ L 1x CHAPS. Then, 10 μ L of each dilution was transferred onto a 96-well plate in duplicates. Additionally, a BSA-dilution series with eight standard points ranging from 0 μ g/10 μ L to 7 μ g/10 μ L was added to create a standard curve for the protein measurement. Then, 200 μ L Bradford reagent (AppliChem, # A6932.0250) were

added to each well to stain the proteins in each sample, followed by incubation for 30 min. at room temperature. Absorbance was measured at a wavelength of 595nm in a TECAN Infinite 200 Pro plate reader. Protein concentration was calculated based on linear regression of BSA-standard-curve.

3.13 cDNA conversion of RNA by reverse transcription

RNA-isolates were incubated for 10 min. at 55°C to dissolve possible RNA-aggregates. Depending on the total concentration of the RNA-isolates between 250ng and 500ng per sample were mixed with 1µl of Random Hexamer Primer and filled up with nuclease free ddH₂O to a total volume of 6µl. This mixture was incubated for 5 min. at 70°C in a PCR-machine, followed by 5 min. cool down on ice. Still on ice, 4µl c-DNA-Synthesis-MasterMix (*Table 9*) were added to each sample, mixed and incubated for 15 min. at 28°C. Then samples were heated for 30 min. to 55°C and finally for 5 min at 85°C. Last but not least, samples were cooled for 5 min. on ice diluted to either 8.3ng/5µl or 20ng/5µl and stored at -20°C.

Table 9 - cDNA synthesis mastermix reagents and volumes for one reaction

2µl	5x Reaction-Buffer
0.25µl	Ribonucleaseinhibitor 40U/µl (Riboblock)
0.25µl	H ₂ O with Inhibitor
1µl	dNTPs 10mM mix
0.5µl	Maxima H Minus Reverse Transcriptase
4µl in total per reaction	

3.14 Real-time qPCR analysis

Quantitative analysis of expression patterns, gene copy numbers and telomeric repeat variants were done in a *BIORAD CFX RT-qPCR system*. Each reaction was done using the *GoTaq® qPCR Master Mix, 2X*. In general, primers were added to each reaction to an end-concentration of 200nM per reaction volume, with a single exception mentioned in *Subchapter 3.14.2*. All results were exported using *BIORAD CFX Maestro* and analysed with *Microsoft Office Excel* and *Graph Pad Prism*.

3.14.1 Expression and gene copy analysis

hTERC expression was analyzed using quantitative real-time PCR (qPCR), targeting specific sequences on cDNA synthesized from RNA isolated from the respective clone variants. 8.3ng cDNA were used per 10µl total reaction volume. Each run was analysed in duplicates in two separate technical repetitions. Expression of hTERC, hTERT and reference gene 36B4 was performed using the primers shown in *Table 10*. Relative expression of hTERC and hTERT was calculated using the *ddCq* method⁴⁴ defining 36B4 as reference gene and the respective untreated parental cell lines as reference samples.

Gene copy analysis of the target genes *hTERC*, *PuroR* and *3'exohTERC* were done analysing the isolated DNA of each sample with an end concentration of 20ng DNA per 10µl total reaction volume. Each run was analysed in duplicates. The untreated controls, WT- and TCA-clones of HT1080 and T98G were analysed in three separate technical repetitions. The untreated controls, WT- and TCA-clones of U2OS and Saos2 were analysed in two separate technical repetitions. TCA-v-, TAA- and TGA-clones of HT1080 and U2OS were analysed once. For quantification of the *hTERC*-gene, the same primers were used as for measurement of the hTERC expression. Primers for quantification of the *PuroR*-gene, the *3'exohTERC*-region and reference gene 36B4 are depicted in *Table 10*.

Reactions of expression and gene-copy analysis were set up using the following program: 2 min. at 95°C, 40 cycles (15 sec. at 95°C and 1 min. at 60°C), 60°C increasing to 95°C with an increment of 0.5°C every 5 sec. Relative gene copy numbers of each target gene were calculated using the *dCq*-method following the formula:

$$\text{relative Gene Copy Number} = 2^{Cq_{\text{target gene}} - Cq_{36B4}}.$$

Table 10 - Overview of primers used for RT-qPCR expression and gene copy analysis

hTERC expression / gene copy primers	Forward-Primer 5'-TCTAACCTAACTGAGAAGGGCGTAG-3' Reverse-Primer 5'-TTTGCTCTAGAATGAACGGTGGAAG-3'
hTERT expression primers	Forward-Primer 5'-ACCGGAAGAGTGTCTGGAGCAA-3' Reverse-Primer 5'-GGGGATGAAGCGGAGTCTGGA-3'
36B4 expression primers	Forward-Primer 5'-CAGCAAGTGGGAAGGTGTAATCC-3' Reverse-Primer 5'-CCCATTCTATCAT CAACGGGTACAA-3'
PuroR gene copy primers	Forward-Primer 5'-GGTCACCGAGCTGCAAGAAC-3' Reverse-Primer 5'-ACACCTTGCCGATGTCTGAG-3'

3'exohTERC gene copy primers	<i>Forward-Primer</i> 5'-CCTTGGCGATGACCTTGAGCAG-3' <i>Reverse-Primer</i> 5'-GTAATACGACTCACTATAGGGC-3'
36B4 gene copy primers	<i>Forward-Primer</i> 5'-TGGTCCTGGTGGGATCCTTTTA-3' <i>Reverse-Primer</i> 5'-CCCATTCTATCATCAACGGGTACAA-3'.

3.14.2 Telomeric repeats variant analysis

Quantification of the telomeric TTAGGG and TCAGGG repeat variants was done with 10ng sample-DNA in a total volume of 10µl per reaction. Each run was analysed in triplicates and each in three separate technical repetitions. However, the isolates of the HT1080-WT from lipofection were only analysed in one run, and the isolates of HT1080-WT clone from the retroviral infection was analysed in two separate runs. Primers used for the quantification of the canonical telomer repeats were forward primer *Telg* and reverse primer *Telc*³⁰ (*Table 11*). For quantification of the TCAGGG repeats, an adaption of the Tel-primer pair was used with the forward primer (*Telg-C*) and a 6-times degenerated reverse primer. As reference gene, 36B4 was measured using the primer pair shown in *Table 11*. Primer assays for the canonical repeats and 36B4 were run with a final concentration of 200nM per reaction, while 200nM of the *Telg-C* primer were combined with 800nM of the degenerated reverse primer for the assay detecting TCAGGG-repeat variants. Both assays were run in a singleplex setting using the following program: 15 min. at 95°C, 2 cycles of 15 sec. at 94°C and 15 sec. at 49°C followed by 40 cycles of 15 secs. at 94°C, 10 secs. at 62°C, 30 sec. at 72°C. The final relative quantity of TCAGGG-repeats (RQ_{final}) was calculated in two steps. First, the relative quantities of both telomeric repeat variants were calculated using the *ddCq*-method⁴⁴, normalizing the quantity of the repeat variants to the reference gene 36B4 and the respective untreated parental cell lines individually, resulting in two separate relative quantities for the TTAGGG- and the TCAGGG-repeat per sample. In a second step, the final quantity of TCAGGG-repeats was calculated using the formula $RQ_{final} = \frac{RQ_{TCAGGG}}{RQ_{TTAGGG}}$.

Table 11 - Overview of primers used for RT-qPCR telomeric repeats variant analysis

TTAGGG repeat primers	<i>Forward-Primer (Telg)</i> 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' (Telg)
	<i>Reverse-Primer (Telc)</i> 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3' (Telc)
TCAGGG repeat primers	<i>Forward-Primer (Telg-C)</i> 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGTC-3' (Telg-C)
	6-times degenerated Reverse-Primer 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCYVACA-3'.
36B4 gene copy primers	<i>Forward-Primer</i> 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGCAGCAAGTGGGAAGGTGTAATCC-3'
	<i>Reverse-Primer</i> 5'-GCCCCGCCCCGCCGCGCCCGTCCCGCCGCCATTCTATCATCAACGGGTACAA-3

3.15 Telomerase activity measurement

The real-time qPCR telomeric repeat amplification protocol (RT-qPCR-TRAP) was used to measure the Telomerase Activity of the clone bulks. This assay was based on the improved TRAP-method first mentioned by Kim and Wu 1997 ⁴⁵. During a primer extension step the telomerase of the isolated cell extracts synthesized telomeric repeats onto the 3'-end of an artificial telomeric oligonucleotide (TS-primer). The resulting extended telomerase products were then quantified in a BIORAD CFX RT-qPCR system, using the initial TS-Primer as forward primer and the ACX-oligonucleotide as reverse primer (*Table 12*). Additionally, each sample was tested with an internal control checking for false-negative results induced by polymerase inhibitors. The internal control used a TSNT-oligonucleotide as template amplified by the forward-primer TS and a K1 reverse-primer. Each sample was diluted to a protein concentration of 150ng/μl in 0,1xCHAPS and measured in duplicates in at least two separate technical repetitions each. As negative control, an aliquot of each sample was denatured at 85°C for 30min. prior to measurement. As positive control, TA cell line K562 was analysed with each run and used as reference for normalization. Each reaction volume contained 4μl diluted protein extract and 4μl Promega *GoTag qPCR Mastermix* spiked with 0.2μl of the respective primer-mixes. Reactions determining the TA and the internal control reactions were run separately in different wells. Each analysis was run under the following conditions: 30 min. at

30°C, 2 min. at 95°C, 40 cycles of (30 secs. at 95°C, 90 secs. at 60°C), 65°C increasing to 95°C with an increment of 0.5°C every 5 secs. Efficiency of this qPCR-TRAP assay was evaluated by measurement of a standard curve by serial dilution of the TSR8 control template in 1:2 dilution steps. As described in Sampl et al. 2012 ³⁹, one TSR8 control template contains sequences of one TS-primer and eight telomeric repeat products. Consequently, the TSR8 standard curve was also used as standard for quantitation of telomerase activity in TPG-unit (Total Product Generated). One TPG-unit corresponds to 600 molecules of TS-Primer extended by at least four telomeric repeats within the incubation time of 30 min. at 30°C. TA of each sample was normalized to reference cell line K562 after each run using the following formula: $relative\ TA = \frac{TA\ sample}{TA\ K562}$. Results were exported using *BIORAD CFX Maestro* and analyzed with *Microsoft Office Excel* and *Graph Pad Prism*.

Table 12 – qPC-TRAP Assay Primers

TS-forward primer	5'-AATCCGTCGAGGCAGTT-3'
ACX- reverse primer	5'-GCGCGG(CTTACC) ₃ CTA ACC-3'
TSNT-substrate	5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT-3'
K1-reverse primer	5'-ATCGCTTCTCGGCCTTTT-3'

3.16 In vitro functionality assay for ectopic hTERC (2qPCR-TRAP)

In a first step, protein lysates of HT1080 and T98G clones overexpressing WT-, TCA- and TCA-v-hTERC variants were run in a qPCR-TRAP reaction. The qPCR-TRAP run was performed as described in *Chapter 3.15* in triplicates and stopped after 35 cycles. The TRAP-products were collected by pooling all three triplicates from each sample. Pooled samples were then diluted in 200µl 0.1x TE-buffer. TRAP-products were then analysed for TTAGGG and TCAGGG copy numbers in a second RT-qPCR-analysis. For quantification of the canonical telomer repeats, forward primer Telg and reverse primer Telc were used as described by Cawthon et al. 2009³⁰ at an end concentration of 200nM each (also see *Table 11*). For quantification of the TCAGGG telomeric repeat variant, a special Telg-C primer was used at a final concentration of 200nM in combination with 800nM of a 6-times degenerated reverse Telg-C primer. Both assays were run in a singleplex setting using the following program: 15 min. at 95°C, 2 cycles of 15 sec. at 94°C and 15 sec. at 49°C followed by 40 cycles of 15 secs. at 94°C, 10 secs. at 62°C, 30 sec. at

72°C. The resulting relative quantities of TCAGGG-repeats of each sample were calculated applying the *ddCq-method*⁴⁴ using the canonical repeats as reference copy number and the respective untreated parental cell lines as reference samples. Results were exported using *BIORAD CFX Maestro* and analysed with *Microsoft Office Excel* and *Graph Pad Prism*.

3.17 Telomere variant fluorescent in situ hybridisation (telo-FISH)

Based on previous publications^{1,27,46} in situ hybridisation of telomers of WT- and TCA-overexpression clone bulks from HT1080 was performed with PNA-probes (PNA Bio INC) binding to TTAGGG and TCAGGG repeat variants. WI38-VA13-2RA cells were used as positive control for co-staining of TTAGGG repeats (red) and TCAGGG repeats (green) and the untreated parental cell line HT1080 as negative control. Each probe contained three consecutive repeats of the either TTAGGG or TCAGGG tagged with either FITC or Cy3. Cells were prepared on a glass-slide using cytospin and fixated in 100% with methanol overnight. Each sample was dehydrated by a sequence of washing steps in 70% Ethanol, 90% Ethanol and 100% Ethanol for 2 min. each. PNA-probes were mixed together and diluted in Hybridization-buffer (10mM TrisCl pH7.5, 70% Formamide, 0.25% Blocking reagent in Maleic Acid Buffer [Roche #11096176001]) to an end-concentration of 66nM. Each sample was then incubated with 15µl of the pNA-solution for 5 min. at 85°C, followed by another incubation for 2 hours in a humidity chamber at room temperature. Samples were then washed two times for each 15 min in PNA wash buffer (140mL formamide, 58mL H₂O, 2mL 1M Tris pH7.5) and another two times for 5 min. each in TBST (100mL 1xTBS + 100 µL Tween-20). DAPI-staining was done by adding 40µl DAPI-solution in TBST (1µg/mL) to each slide followed by incubation for 15 min. at room temperature. Samples were washed again for 5 min. in TBST and two times for 5 min. each in ddH₂O. Finally, stained samples were fixed in with VECTASHIELD® mounting medium and stored at 4°C protected from light.

3.18 Microscopy

Cell culture growth was observed using Phase contrast and Fluorescence microscopy on a *Nikon Eclipse Ti inverse Fluorescence Microscope*. The Telo-FISH experiment was observed on

an *Olympus IXplore Spinning Disk Confocal system*. All pictures were edited using *Adobe Photoshop 2021* for cropping, inserting a scale reference and layout formation.

3.19 Statistics

Statistical analyses were performed using GraphPad Prism (version 8.0.1). The Shapiro-Wilk test was employed to assess Gaussian distribution. Variance distribution was evaluated using the F-test. For datasets with normally distributed variances, an unpaired t-test was conducted, whereas Welch's t-test was applied when variances were non-normally distributed. A significance level of $p < 0.05$ was set for both tests. Information on repetition counts and sample sizes is provided in the corresponding result descriptions.

4 Results

4.1 Puromycin resistance analysis

First, the puromycin concentration for later selection experiments of plasmids in cell clones was established. Therefore, puromycin resilience of eight cell lines (HT1080, HeLa, SW480, SW480-LT, T98G, U2OS, Saos2, YT-BO) was analysed. In a preliminary experiment, puromycin resistance was tested for U2OS and Saos2 at puromycin concentrations ranging between 0 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$. The preliminary experiment was stopped prematurely as most cell did not survive incubation for 5 days at these puromycin levels (*Figure S 1*). Subsequently, concentrations were adjusted as described below.

The cell lines were exposed to eight different puromycin concentrations (ranging 0.1 to 5 $\mu\text{g/mL}$). In detail, puromycin was added to their respective media and exposed to cells for 5 days, as literature suggested ⁴⁷. The optimal puromycin dose to use for selection experiments is typically the lowest antibiotic concentration that results in complete cell death within the timeframe of the selection interval. A control growth experiment lacking puromycin was included for each cell line to serve as positive control for the puromycin selection. Puromycin concentrations and incubation time were defined to span the interval of puromycin concentrations used in similar selection experiments found in previous published studies ^{1,48,49}. Relative cell numbers after puromycin exposure were assessed using a Sulphorhodamine B (SRB) assay measuring absorbance of SRB bound to cellular proteins after antibiotic treatment at 570nm. The absorption value indicates the quantity of cell mass in a sample from which the amount of remaining viable cells can then be estimated.

As *Figure 4* shows, puromycin proved to efficiently decrease relative cell number already at low concentrations in all cell lines. When comparing cells incubated at 0.1 $\mu\text{g/mL}$ Puro with the control group, a drop in relative cell number was observed for all cell lines except YT-BO. This trend continued with increasing puromycin concentrations of up to 1 $\mu\text{g/mL}$ as cell number gradually decreased further. When drastically increasing the antibiotic pressure from 1 $\mu\text{g/mL}$ to 5 $\mu\text{g/mL}$ Puro, hardly any additional decrease of cell number was visible in cell lines SW480, SW480-LT, HeLa, HT1080, T98G, U2OS and Saos2. In comparison, relative cell number of YT-BO stagnated less drastically, beginning at 0.2 $\mu\text{g/mL}$ puromycin and continuing with every further increase of puromycin. Even at the threshold of 1 $\mu\text{g/mL}$ puromycin, relative cell

number of YT-BO continued to drop. At a puromycin concentration of 5 $\mu\text{g}/\text{mL}$, YT-BO still displayed a higher relative cell number, when compared to all other cell lines.

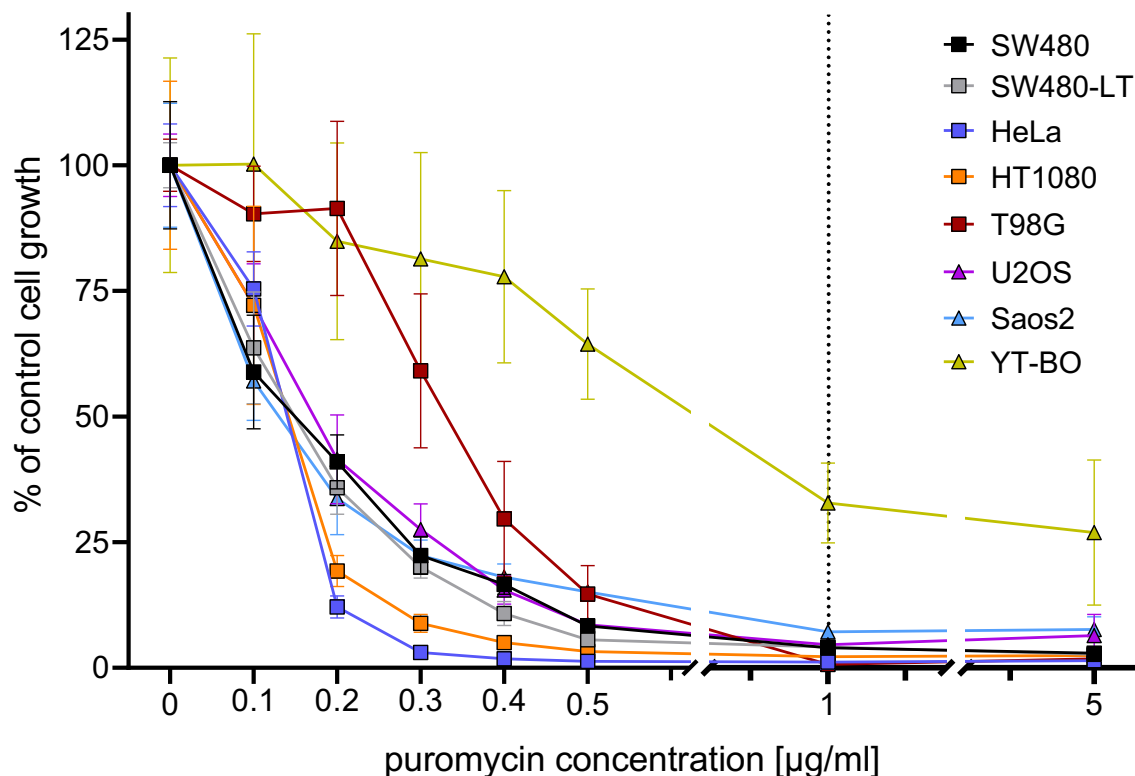


Figure 4 – Cytotoxicity (SRB) assay of TA cell lines SW480, SW480-LT, HeLa, HT1080, T98G (squares) and ALT cell lines U2OS, Saos2, YT-BO (triangles) at eight different concentrations of puromycin performed as single experiment. Each value depicts the mean relative cell number of six biological replicates ($n=6$), calculated by $\text{Absorbance sample} / \text{Mean Absorbance untreated control} \times 100$, adapted from Orellana & Kasinski 2016⁵⁰. Absorbance values are depicted separately in Chapter 8 **Figure S 1**. Error bars show the standard deviation (SD). The most efficient reduction of cell growth for all eight cell lines was marked with a dotted line at a concentration of 1 $\mu\text{g}/\text{mL}$ puromycin.

Cell lines SW480, SW480-LT, HeLa, HT1080, T98G, U2OS and Saos2 proved more sensitive to puromycin, uniformly showing a drastic drop of relative cell number up to a threshold value of 1 $\mu\text{g}/\text{mL}$ puromycin. The uncharacteristic lack of decrease between 0.1 und 0.2 $\mu\text{g}/\text{mL}$ puromycin in T98G cells could be an effect of the high standard deviation between the replicates at that measurement point.

In summary, apart from YT-BO, cell lines SW480, SW480-LT, HeLa, HT1080, T98G, U2OS and Saos2 showed very similar resilience levels to puromycin treatment. For this study, a concentration of 1 µg/mL puro proved to be sufficient to effectively reduce cell numbers until almost no viable cells could be measured in all TA cell lines as well as ALT cell lines U2OS and Saos2. Rest signal could be explained by presence of a small number of surviving cells. It is possible that a longer treatment with puromycin at that concentration would have resulted in a complete reduction of viable cells in these cell lines. Therefore, a threshold of 1 µg/mL puromycin was chosen for selection of puromycin resistant overexpression clones later on.

ALT cell line YT-BO was excluded from the following experiments due to its elevated ability to survive exposure to the shown puromycin concentrations.

4.2 Expression analysis of hTERC and hTERT in parental cell lines

A screening of endogenous hTERC and hTERT expression levels of the remaining seven cell lines was performed. The aim was to compare endogenous transcript expression thresholds to pick the most interesting cell lines for hTERC overexpression experiments. Expression patterns of both major telomerase subunits were analysed using RT-qPCR. Relative gene expression of both target transcripts was normalized to reference gene 36B4 and cell line HT1080.

TA cell lines HT1080, HeLa, SW480, SW480-LT and T98G showed varying but strong expression of both major telomeric subunits (*Figure 5*). ALT cell line U2OS displayed significantly lower levels of both telomeric subunits when compared to most other cell lines. hTERC expression of ALT cell line Saos2 was shown to be at a similar level as all five TA cell lines. While hTERT levels of Saos2 had been close to the detection limit, with one replicate showing no hTERT expression signal.

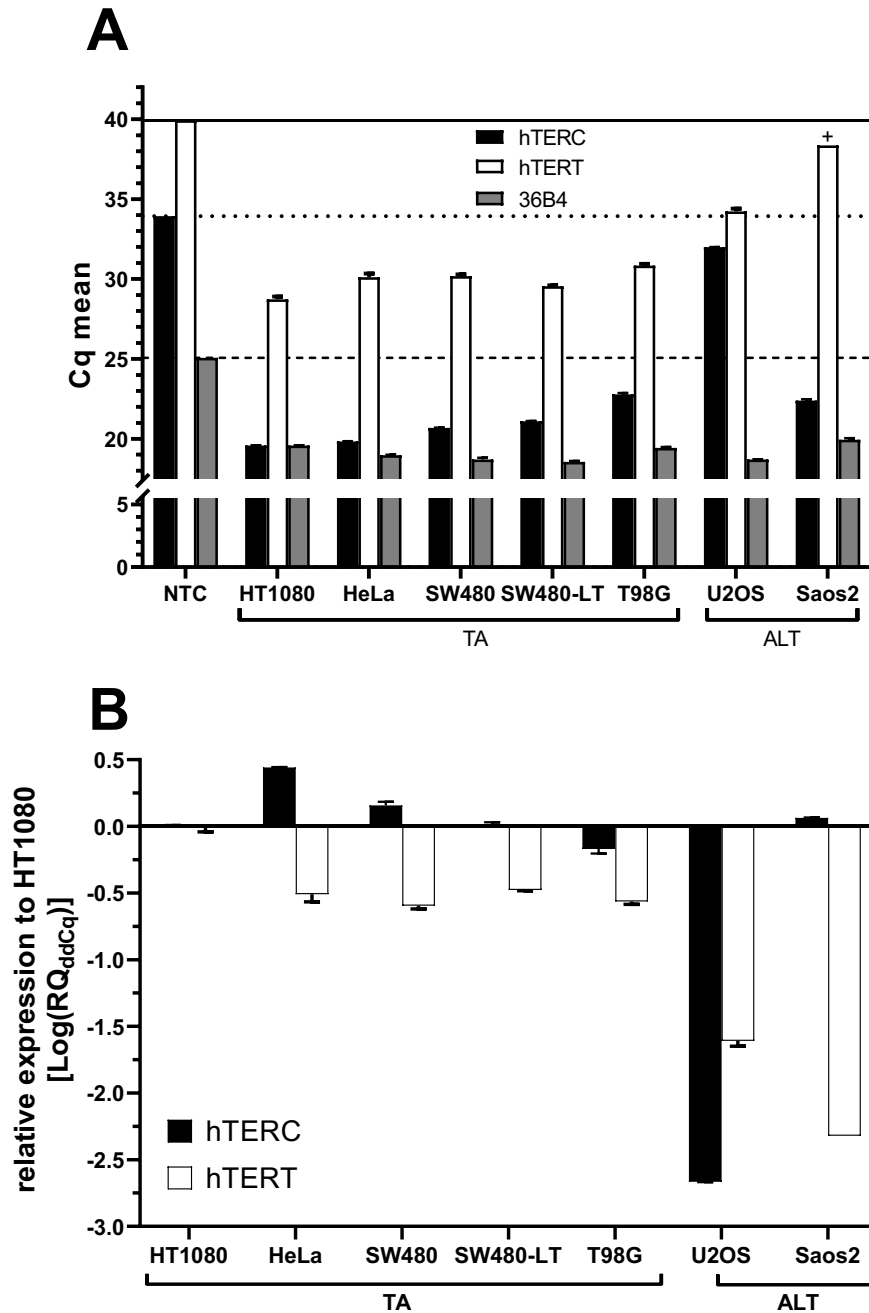


Figure 5 - Endogenous expression of telomerase subunits *hTERC* and *hTERT* in TA cell lines HT1080, HeLa, SW480, SW480-LT, T98G and ALT cell lines U2OS, Saos2. **(A)** Mean Cq values of *hTERC*, *hTERT* and 36B4 expression analysis including non-template controls (NTC). Error bars show standard deviation (SD). NTC values show thresholds for false-positive signals, marked with a dotted line (*hTERC*), a straight line (*hTERT*) and a dashed line (36B4). In Saos2, an *hTERT* expression signal could be detected in only one triplicate (Cq 38.36, marked with +). Target gene *hTERT* was analysed in triplicates ($n=3$), *hTERC* and 36B4 in duplicates ($n=2$). No technical replicates were made for the NTCs ($n=1$). This RT-qPCR analysis was performed as single experiment. **(B)** Mean relative expression shown as Log10 transformed Relative Quantity (RQ) normalized on reference cell line HT1080 and reference gene 36B4 (ddCq). Error bars show SD.

In detail, TA cell line HeLa displayed the highest endogenous hTERC expression of all seven cell lines. The expression of hTERC being 2.8 ± 0.013 fold higher than of reference cell line HT1080, while expression of the catalytic subunit hTERT was lower with a relative quantity of 0.3 ± 0.034 (Figure 5 and Table 13).

Table 13 - Relative gene expression of hTERC and hTERT shown as mean RQ normalized to reference gene 36B4 and reference cell line HT1080 (ddCq)

TMM	Cell line	hTERC RQ _{mean} $\pm$ SD	hTERT RQ _{mean} $\pm$ SD
TA	HT1080 ^a	1.0 ± 0.017	1.0 ± 0.076
	HeLa	2.8 ± 0.013	0.3 ± 0.034
	SW480	1.4 ± 0.061	0.3 ± 0.012
	SW480-LT	1.0 ± 0.027	0.3 ± 0.003
	T98G	0.7 ± 0.039	0.3 ± 0.012
ALT	U2OS	$0.002 \pm 0.1 \times 10^{-4}$	0.02 ± 0.002
	Saos2 ^b	1.2 ± 0.008	0.005

^a Reference cell line

^b hTERT RQ is shown as single value, as an hTERT signal was detected in only one of three triplicates

TA cell line SW480 also showed a similar expression pattern with a relative quantity of 1.4 ± 0.061 regarding hTERC expression, and 0.3 ± 0.012 regarding hTERT expression.

TA cell line SW480-LT showed no relative increase in hTERC expression with a relative quantity of 1.0 ± 0.027 , and a relative decrease of hTERT expression concerning hTERT at 0.3 ± 0.003 , when compared to the reference cell line HT1080.

On the other hand, cell line T98G displayed the lowest endogenous hTERC expression of all TA cell lines with a relative quantity of 0.7 ± 0.039 . Endogenous hTERT level of cell line T98G remained at a relative quantity of 0.3 ± 0.012 , similar to cell lines HeLa, SW40 and SW40-LT.

TA cell line HT1080, used a reference cell line, displayed a very similar hTERC expression level compared to SW480-LT. Moreover, this cell line showed the highest endogenous expression of the catalytic subunit. The hTERT expression of HT1080 is approximately three times higher than of the other five TA cell lines.

Regarding the ALT cell lines, U2OS displayed drastically decreased levels of both subunits standing at a relative quantity of $0.002 \pm 0.1 \times 10^{-4}$ concerning hTERC expression, and 0.02 ± 0.002 concerning hTERT expression.

ALT cell line Saos2 on the other hand, showed a similar hTERC expression level as reference cell line HT1080 with a relative quantity of 1.2 ± 0.008 . While hTERT expression had hardly been detectable showing a relative quantity of 0.005, while one replicate did not show any hTERT signals.

Based on data gained from this experiment, HT1080 and T98G were selected as TA cell lines and U2OS and Saos2 as ALT cell lines for the subsequent hTERC overexpression experiments. HT1080 was considered an interesting candidate cell line, as it was already used in similar studies^{1,31} as well as proved to have strong hTERT expression. A high level of hTERT expression might be beneficial for incorporation of overexpressed ectopic variant repeats into the telomers. T98G on the other hand was chosen for its low amount of native hTERC expression. Low amounts of native hTERC could be an advantage when overexpressing ectopic hTERC variants, by allowing a higher proportion of ectopic hTERC to be expressed in the cell. U2OS and Saos2 were chosen as counterparts to HT1080 and T98G for their use of the ALT as telomer maintenance mechanism.

4.3 Introduction and selection of clones overexpressing ectopic hTERC

pBABE-Puro-U3-hTERC-500 plasmids overexpression-plasmids containing wild-type and variant hTERC sequences (*Table 14*) were introduced to all TA cell lines HT1080, T98G and ALT cell lines U2OS and Saos2. The plasmids were introduced to the cells using Lipofection (Lipo) and Retroviral Infection (RV). After incubation for 48-64 hours, antibiotic pressure was applied to treated cells over a time period of 2 to 12 weeks to select for cells stably overexpressing ectopic hTERC. A puromycin resistance gene linked to the ectopic hTERC gene was used as selection marker for clones that integrated and expressed the target genes.

Table 14 – Overview regarding established clones of cell lines HT1080, T98G, U2OS and Saos2 with WT and hTERC variant constructs

hTERC variants	HT1080		T98G		U2OS		Saos2	
	RV	LIPO	RV	LIPO	RV	LIPO	RV	LIPO
WT	x*	x	x	x	x*	x	N/S	x
TCA	x*	x	N/S	x	x*	x	N/S	x
TCA-v	x	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TAA	x	N/A	N/A	N/A	x	N/A	N/A	N/A
TGA	x	N/A	N/A	N/A	x	N/A	N/A	N/A

x – successfully selected clones

x* – two independent experiments performed

N/S – stable clones could not be successfully selected

N/A – not chosen for Lipofection/Retroviral infection experiments

LIPO – Lipofection

RV – Retroviral Infection

A total of 22 clones stably expressing the introduced puromycin resistance marker were selected after introduction of overexpression plasmids to the cells (*Table 7* and *Table 14*). To keep the number of different clones within a reasonable amount for this study, not every hTERC-variant was introduced to each cell line. Hence, WT- and TCA-hTERC variants were introduced to cell lines HT1080, T98G, U2OS and Saos2 using Lipofection and Retroviral Infection.

hTERC- variants TCA-v, TAA and TGA were introduced to HT1080 and U2OS only using retroviral infection in a second transduction experiment.

T98G cells transduced with the WT-variant, and Saos2 cells transduced with the WT- and TCA-variant using Retroviral Infection did not survive the selection experiments with puromycin and had therefore been excluded from subsequent characterisation experiments.

4.4 Transient hTERC overexpression in HT1080 and T98G clones

Transient overexpression of hTERC in Lipofection clones was analysed by measurement of total hTERC expression in HT1080 and T98G cells 48 hours after transfection. Samples of WT- and TCA-clones of HT1080 and T98G were analysed and compared to the endogenous hTERC expression of their respective untreated parental cell lines.

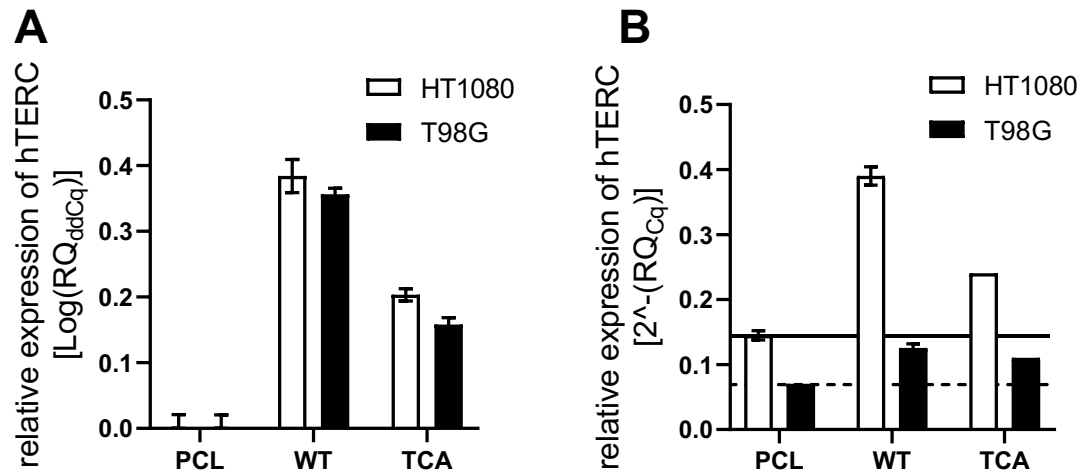


Figure 6 – Total expression of hTERC in clones of TA cell lines HT1080 and T98G measured two days after lipofection with overexpression plasmids for the WT- and the TCA-variant. The expression of the target gene hTERC and reference gene 36B4 was measured in duplicates (n=2) in one experiment. Error bars show standard deviation. **Chart A** - Log10 values of hTERC expression relative to the untreated parental cell lines (PCL) of the respective cell lines and normalized to reference gene 36B4 using the ddCq method. This graph highlights the increase of total hTERC expression after introduction of the plasmids compared to the PCL. **Chart B** - hTERC expression relative to housekeeping gene 36B4 using the dCq method. This graph gives an overview of the differences in hTERC expression of all four analysed clones as well as their PCLs. Both straight and dotted line highlight the respective endogenous expression levels of hTERC measured in the untreated controls (PCLs).

A significant increase of total hTERC expression levels was detectable in both clone-variants of HT1080 and T98G (Figure 6A and Table 15). WT-variants of HT1080 and T98G displayed an increase of 2.4 ± 0.141 and 2.3 ± 0.048 regarding hTERC expression, when compared to the respective untreated controls. TCA-variants also showed a significant increase in hTERC expression at a magnitude of 1.6 ± 0.034 in HT1080, and 1.4 ± 0.034 in T98G. The strength of increase seen in both cell lines displayed a very similar pattern with the most drastic effect in the WT-variants.

Table 15 - hTERC expression of 48 hours after Lipofection of HT1080 and T98G cells relative to parental cell lines (ddCq)

Variant	HT1080 ¹ RQ_{mean} ± SD	T98G ² RQ_{mean} ± SD
PCL	1.0 ± 0.049	1.0 ± 0.047
WT	2.4 ± 0.141	2.3 ± 0.048
TCA	1.6 ± 0.034	1.4 ± 0.034

¹ normalized on parental cell line HT1080 and reference gene 36B4

² normalized on parental cell line T98G and reference gene 36B4

A significant difference in overall hTERC expression strength was detectable between both cell lines in the overexpression variants and the untreated controls as shown in *Figure 6B*. Clones of HT1080 uniformly showed a two to three times higher total hTERC expression level, than clones of T98G.

In conclusion, clones of HT1080 and T98G displayed increased expression of hTERC within two days after the lipofection experiments were completed. Consequently, transient hTERC overexpression was successfully induced by introducing overexpression plasmids carrying ectopic hTERC-variants WT- and TCA- to the cells by lipofection.

4.5 Stable hTERC overexpression in selected clones

Total hTERC expression was analysed of clones from cell lines HT1080, T98G, U2OS and Saos2 after treatment with puromycin using real time qPCR. Clones which displayed a significant increase in total hTERC expression after surviving the puromycin selection period were considered as stable hTERC overexpression clones.

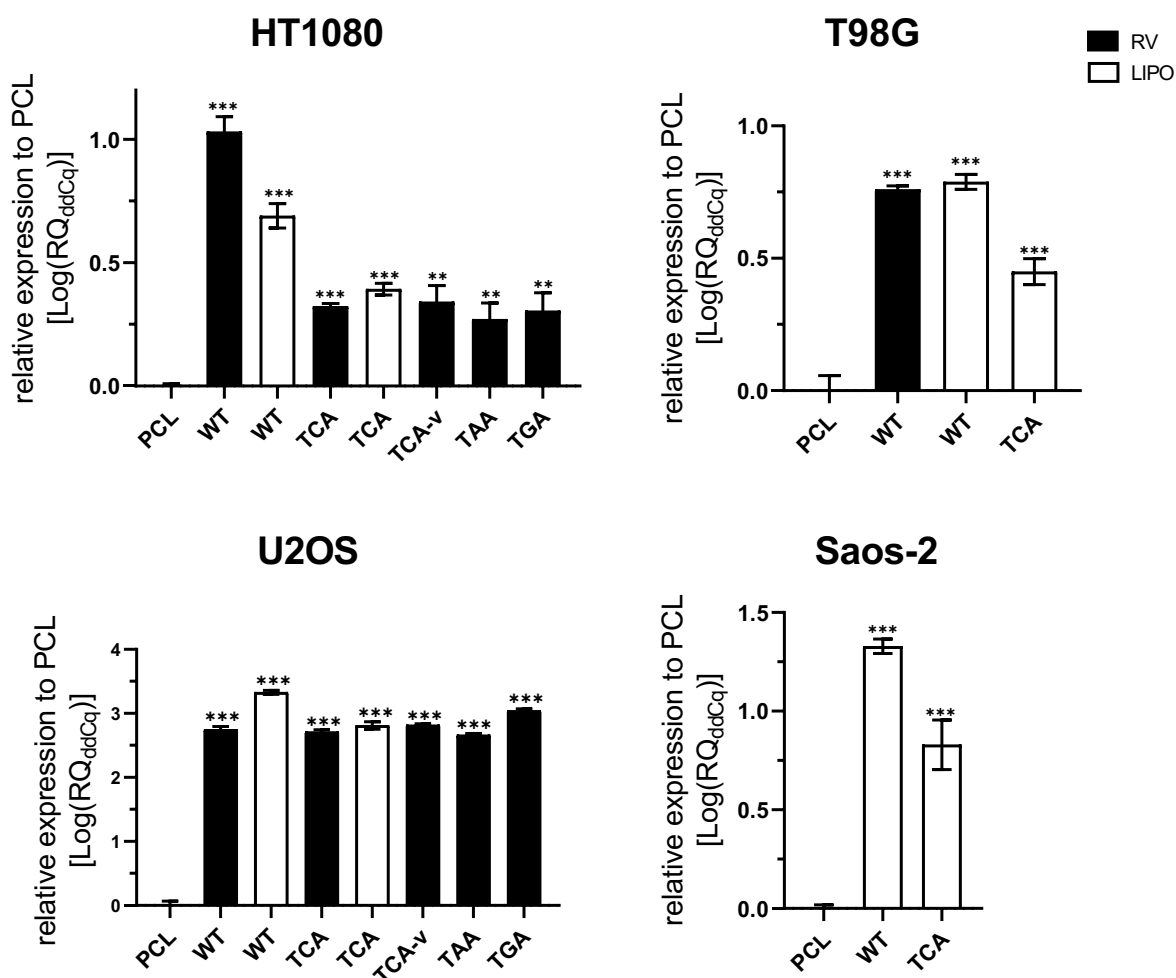


Figure 7 - Total hTERC expression in clones of cell lines HT1080, T98G, U2OS and Saos2 after puromycin treatment. The expression of the target gene hTERC and reference gene 36B4 was measured in duplicates in three independent RT-qPCR experiments (n=6). Error bars show standard deviation. Expression levels are shown as Log10 values of hTERC expression relative to the untreated controls (PCL) of the respective cell lines and normalized to reference gene 36B4 using the ddCq method. Significances in hTERC expression increase were calculated between the clones and their respective parental cell lines using t-Test and Welch's-Test.

Table 16 - Overview of total expression of hTERC of puromycin treated clones relative to parental cell lines

	Total hTERC expression ^a RQ _{mean} ± SD			
hTERC variant	HT1080	T98G	U2OS	Saos2
PCL	1.0 ± 0.018	1.0 ± 0.096	1.0 ± 0.184	1.0 ± 0.036
WT ^{RV}	7.7 ± 0.272	5.8 ± 0.147	1328 ± 30.3	-
WT ^{LIPO}	4.9 ± 0.483	6.2 ± 0.345	2140 ± 133.3	23.1 ± 0.005
TCA ^{RV}	2.1 ± 0.047	-	519 ± 30.2	-
TCA ^{LIPO}	2.5 ± 0.117	2.8 ± 0.277	652 ± 74.5	7.0 ± 1.769
TCA-v ^{RV}	2.2 ± 0.282	-	660 ± 22.9	-
TAA ^{RV}	1.9 ± 0.249	-	462 ± 18.6	-
TGA ^{RV}	2.0 ± 0.294	-	1110 ± 57.4	-

^a Normalized on respective parental cell line and reference gene36B4 (ddCq)

RV Clones generated by Retroviral Infection

LIPO Clones generated by Lipofection

Significant increase in hTERC expression was detectable in every analysed clone as depicted in *Figure 7*, when compared to the respective parental cell lines. The most prominent effect was seen in clone variants expressing the ectopic WT-construct throughout all cell lines.

When looking into the relative quantities of hTERC expression (*Table 16*), increase of expression ranged between 1.9 ± 0.249 and 7.7 ± 0.272 in HT1080 clones. Displaying the most prominent increase in the WT-RV clone with 7.7 ± 0.272 , followed by the WT-LIPO clone variant with 4.9 ± 0.483 . A less drastic, but still significant increase of hTERC expression was observed for variants TCA-RV, TCA-LIPO, TCA-v, TAA and TGA, with relative quantities ranging between 1.9 ± 0.249 and 2.5 ± 0.117 .

A similar pattern was observed in clones of T98G, which displayed a relative increase of 5.8 ± 0.147 and 6.2 ± 0.345 in the respective WT-variants. The increase in the TCA-LIPO variant being less drastic at 2.8 ± 0.27 , when compared to the untreated control (PCL).

A massive increase in hTERC expression was detected in all overexpression clones of U2OS. The relative increase ranging from 462 ± 18.6 to a 2140 ± 133.3 , when compared to the untreated control, which displayed very low endogenous hTERC expression levels (see *Chapter 4.2*). The most prominent effects were seen again in both WT-clones and the TGA-clone. The WT-RV and the WT-LIPO clones showed relative quantities of 1328 ± 30.3 and 2140 ± 133.3 ,

respectively, while the TGA clone had a relative quantity of 1110 ± 57.4 . A smaller increase was seen in clone variants TCA-RV, TCA-LIPO, TCA-v and TAA ranging between 462 ± 18.6 and 660 ± 22.9 .

Saos2 showed an overall strong increase in hTERC expression with a relative quantity of 23.1 ± 0.005 in the WT-LIPO clone, and 7.0 ± 1.769 in the TCA-LIPO clone.

In general, all tested clones displayed a significant increase in total hTERC expression after puromycin treatment and can therefore be considered stable hTERC overexpression clones.

4.6 Gene copy numbers of target genes hTERC, PuroR and 3'exohTERC

Stable integration of the ectopic target genes into the genomes introduced to the cells was a prerequisite for successful stable overexpression in selected clones. A relative gene copy number analysis was performed from DNA samples of the stable overexpression clones to test whether introduction of the overexpression plasmids led to incorporation of the target sequences hTERC, PuroR and 3'exohTERC into their genomes. The analysis was performed using RT-PCR assays. Relative gene copy numbers were calculated relative to reference gene 36B4 using the dCq-method.

The PuroR gene is located upstream of the ectopic hTERC gene on the overexpression plasmid, carrying the necessary resistance to survive antibiotic treatment with puromycin over a longer period of time. The 3'exohTERC region is an additional unique sequence located downstream of the ectopic hTERC gene on the pBABE-Puro-U3-hTERC-500 plasmid (*Figure 8* and *Figure S 2*). Both, PuroR and 3'exohTERC do not exist in the endogenous sequences of the human genome.

Following the hypothesis that all stable clones have integrated the target genes into their genomes, an increased amount of hTERC gene copies should be found in selected clones when compared to their parental cell lines. As PuroR and 3'exohTERC are not endogenous genomic regions, these target genes are expected to have integrated into the genomes of selected overexpression clones only.

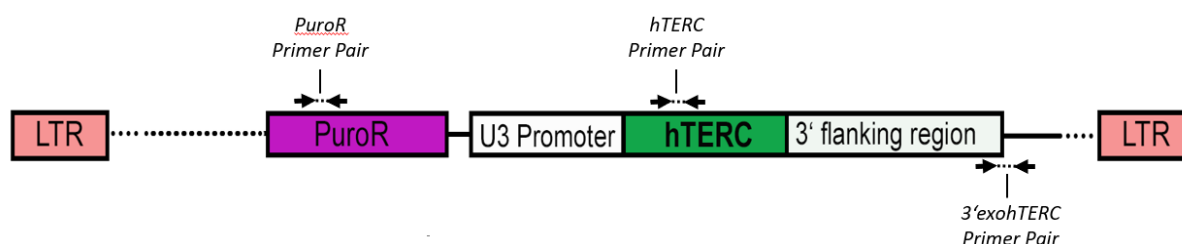


Figure 8 – Schematic excerpt of the pBABE-Puro-U3-hTERC-500 plasmid showing the Puromycin resistance gene located upstream of the ectopic hTERC gene linked to a U3-promoter, initially created by Fu and Collins ⁴². Target regions of primer pairs used for gene copy analysis of PuroR, hTERC and 3'exohTERC were marked. The 3'exohTERC region is a unique sequence used to indirectly quantify the integration of the ectopic hTERC gene into the genomes of the clones. The 3'exohTERC region is located at the end of the 3' flanking region and spans into the pBABEpuro backbone of the plasmid construct. On both ends of the plasmid regions shown, long terminal repeats are marked as starting and endpoints of integration using retroviral infections.

As Figure 9A and Table 17 show below, an increase in relative hTERC gene copy number was seen in 16 overexpression clones, when compared to the endogenous hTERC gene copy numbers of the parental cell lines. No increase was seen in variants WT-LIPO, TCA-LIPO and TAA of cell line HT1080.

Moreover, a high standard deviation was observed concerning hTERC gene copy results. This issue remained present despite repeated analysis of the selected clone variants. Only few variants showed standard deviations within an acceptable threshold. Overall, reliable quantification of the total hTERC gene copy numbers was therefore not possible based on the data gained from this experimental setup.

In contrast, quantification of PuroR and 3'exohTERC gene copy numbers were more reliable. (Figure 9B/C and Table 17). Regarding resistance marker PuroR, no gene copies were detected in the parental cell lines HT1080, T98G and U2OS meeting the expectations as negative controls (Figure 9B and Table 17). A relative gene copy number of 0.5 ± 0.09 of the PuroR gene was detected in the DNA of the parental cell line of Saos2, indicating a potential contamination issue of the sample. Relative amount of PuroR gene copies in the overexpression clones ranged between 0.2 ± 0.05 and 6.0 ± 0.4 . The highest relative copy number was found in Saos2-WT-LIPO and Saos2-TCA-LIPO clones, respectively. While a relative gene copy number of 0.2 ± 0.05 was detected in U2OS clone WT-RV. Overall, PuroR copies were identified in the genomes of all selected overexpression clones.

No gene copies of the exogenous 3'exohTERC region were found in the parental cell lines of HT1080, T98G, U2OS and Saos2 (*Figure 9C* and *Table 17*). Whereas relative quantity of 3'exohTERC ranged between 0.5 ± 0.1 and 23.1 ± 2.9 in the overexpression clones. Clones of TA cell lines HT1080 and T98G uniformly integrated a relative quantity of up to 2.0 ± 0.3 gene copies. ALT cell lines U2OS showed slightly higher relative number of integrations ranging between 1.9 ± 0.2 and 3.9 ± 0.7 . Saos2-WT and -TCA clones displayed the highest relative gene copy numbers with 18.8 ± 2.2 and 23.1 ± 2.9 , respectively.

In summary, gene copy analysis revealed that all analyzed overexpression clones integrated various copies of the PuroR and 3'exohTERC gene sequences from the introduced plasmid sequences into their genomes. Although the replication issue of the hTERC analysis made it hard to gain reliable information of the exact hTERC gene quantities in the DNA samples, data suggests an increase in hTERC gene copies in most overexpression clones.

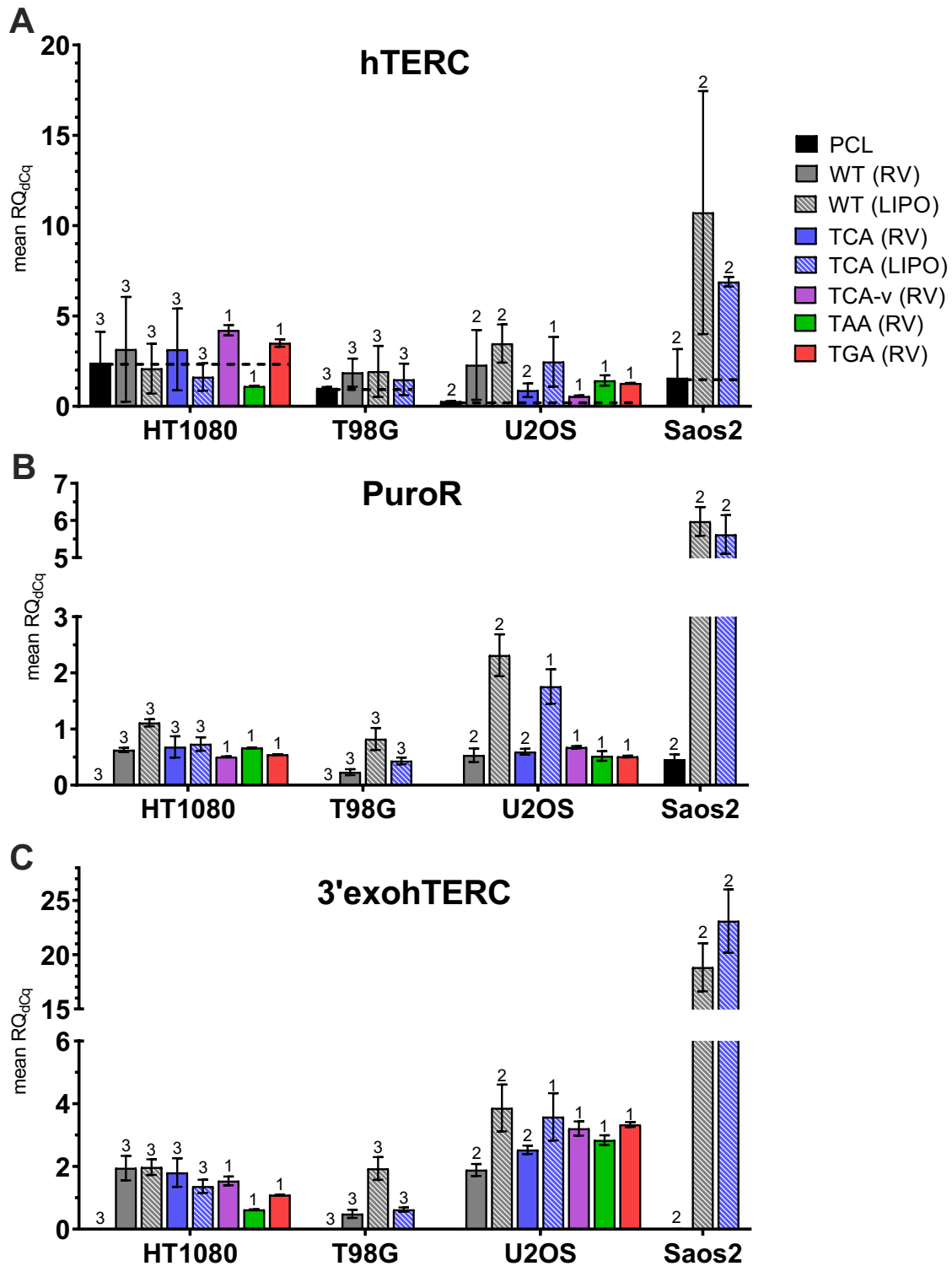


Figure 9 - Relative Gene copy numbers of target genes hTERC (A), PuroR (B) and 3'exohTERC (C) in genomic DNA of stable hTERC overexpression clones. Untreated parental cell lines were included as negative controls. Gene copy numbers were determined relative to reference gene 36B4 using the dCq method. Error bars show standard deviations. Due to high standard deviations of the hTERC gene copy assays results, RT-qPCR analysis was repeated up to three times depending on clone variant ($n = x*2$). Numbers written over error bars represent number of repetitions performed. Dotted lines in Figure 9A represent threshold of endogenous hTERC copy numbers measured in parental cell lines.

Table 17 - hTERC, PuroR and 3'exohTERC gene copy results in stable clones

	hTERC RQ _{mean} $\pm$ STD	PuroR RQ _{mean} $\pm$ STD	3'exohTERC RQ _{mean} $\pm$ STD	Repetitions *
- HT1080 -				
PCL	2.4 $\pm$ 1.7	0	0	3
WT^{RV}	3.2 $\pm$ 2.9	0.6 $\pm$ 0.04	1.9 $\pm$ 0.4	3
WT^{LIPO}	2.1 $\pm$ 1.4	1.1 $\pm$ 0.06	2.0 $\pm$ 0.3	3
TCA^{RV}	3.1 $\pm$ 2.3	0.7 $\pm$ 0.2	1.8 $\pm$ 0.5	3
TCA^{LIPO}	1.6 $\pm$ 0.8	0.7 $\pm$ 0.1	1.4 $\pm$ 0.2	3
TCA-v^{RV}	4.2 $\pm$ 0.3	0.5 $\pm$ 0.007	1.5 $\pm$ 0.1	1
TAA^{RV}	1.1 $\pm$ 0.03	0.7 $\pm$ 0.007	0.6 $\pm$ 0.007	1
TGA^{RV}	3.5 $\pm$ 0.01	0.5 $\pm$ 0.007	1.1 $\pm$ 0.01	1
- T89G -				
PCL	1.0 $\pm$ 0.1	0	0	3
WT^{RV}	1.9 $\pm$ 0.8	0.2 $\pm$ 0.05	0.5 $\pm$ 0.1	3
WT^{LIPO}	1.9 $\pm$ 1.4	0.8 $\pm$ 0.2	1.9 $\pm$ 0.4	3
TCA^{RV}	-	-	-	-
TCA^{LIPO}	1.5 $\pm$ 0.9	0.4 $\pm$ 0.06	0.6 $\pm$ 0.07	3
TCA-v^{RV}	-	-	-	-
TAA^{RV}	-	-	-	-
TGA^{RV}	-	-	-	-
- U2OS -				
PCL	0.3 $\pm$ 0.02	0	0	2
WT^{RV}	2.3 $\pm$ 1.9	0.5 $\pm$ 0.1	1.9 $\pm$ 0.2	2
WT^{LIPO}	3.5 $\pm$ 1.1	2.3 $\pm$ 0.4	3.9 $\pm$ 0.7	2
TCA^{RV}	0.9 $\pm$ 0.4	0.6 $\pm$ 0.05	2.5 $\pm$ 0.1	1
TCA^{LIPO}	2.5 $\pm$ 1.4	1.8 $\pm$ 0.3	3.6 $\pm$ 0.8	2
TCA-v^{RV}	0.6 $\pm$ 0.04	0.7 $\pm$ 0.02	3.2 $\pm$ 0.2	1
TAA^{RV}	1.4 $\pm$ 0.3	0.5 $\pm$ 0.08	2.8 $\pm$ 0.2	1
TGA^{RV}	1.3 $\pm$ 0.01	0.5 $\pm$ 0.01	3.3 $\pm$ 0.08	1
- Saos2 -				
PCL	1.6 $\pm$ 1.6	0.5 $\pm$ 0.09	0 $\pm$ 0.005	2
WT^{RV}	-	-	-	-
WT^{LIPO}	10.7 $\pm$ 6.7	6.0 $\pm$ 0.4	18.8 $\pm$ 2.2	2
TCA^{RV}	-	-	-	-
TCA^{LIPO}	6.9 $\pm$ 0.3	5.6 $\pm$ 0.5	23.1 $\pm$ 2.9	2
TCA-v^{RV}	-	-	-	-
TAA^{RV}	-	-	-	-
TGA^{RV}	-	-	-	-

* each repetition was performed in 2 technical replicates

4.7 Influence of hTERC variant overexpression on telomerase activity

The telomerase enzyme is composed of both the hTERC RNA and the catalytic subunit hTERT. Both subunits are required for the telomerase enzyme to be fully functional. Furthermore, both subunits have been shown to have restrictive effects on telomerase activity^{51,52}. Therefore, it was hypothesized that the overexpression of the RNA subunit hTERC might also influence the activity of the telomerase complex in cancer cell lines. To test this hypothesis, the telomerase activity of all clones of TA cell lines HT1080 and T98G was analysed using telomerase repeated amplification protocol assays (RT-qPCR-TRAP). Cell line K562 was used as positive control, as well as reference cell line for normalization of telomerase activities.

In cell line HT1080, drastic increases in telomerase activity were observed in clones WT-RV and TGA and TCA-v (*Figure 10A*). A less prominent increase was detected in clone TAA. Indications of increased Telomerase activity were also found in the WT-LIPO and TCA-RV variants. TA evaluation also suggested a slight decrease in the TCA-LIPO variant of HT1080.

Regarding cell line T98G, strong increases of TA activity were observed in both WT-variants. Indications for an even higher increase were observed in the TCA-LIPO variant. However, high standard deviations rendered the result unreliable. Overall, the high standard deviations observed in the TPG evaluation made it difficult to draw definitive conclusions from the TPG data.

To address this issue, the TPG results were normalized to the positive control K562 for each independent qPCR-TRAP run, thereby reducing the standard deviations (*Figure 10B*). A significant increase in relative telomerase activity was observed in 6 out of 7 HT1080 clone variants. The only exception was the TCA-LIPO variant, which showed a slight but significant decrease in relative telomerase activity.

In T98G clone variants WT-RV and WT-LIPO, a significant increase of relative telomerase activity was observed. TCA-LIPO variant of T98G displayed no significant increase. Despite normalization to control cell line K562, the high standard deviation still rendered the results of this sample unreliable.

In summary, telomerase activity was significantly increased in 8 out of 10 variants of HT1080 and T98G stable hTERC overexpression clones.

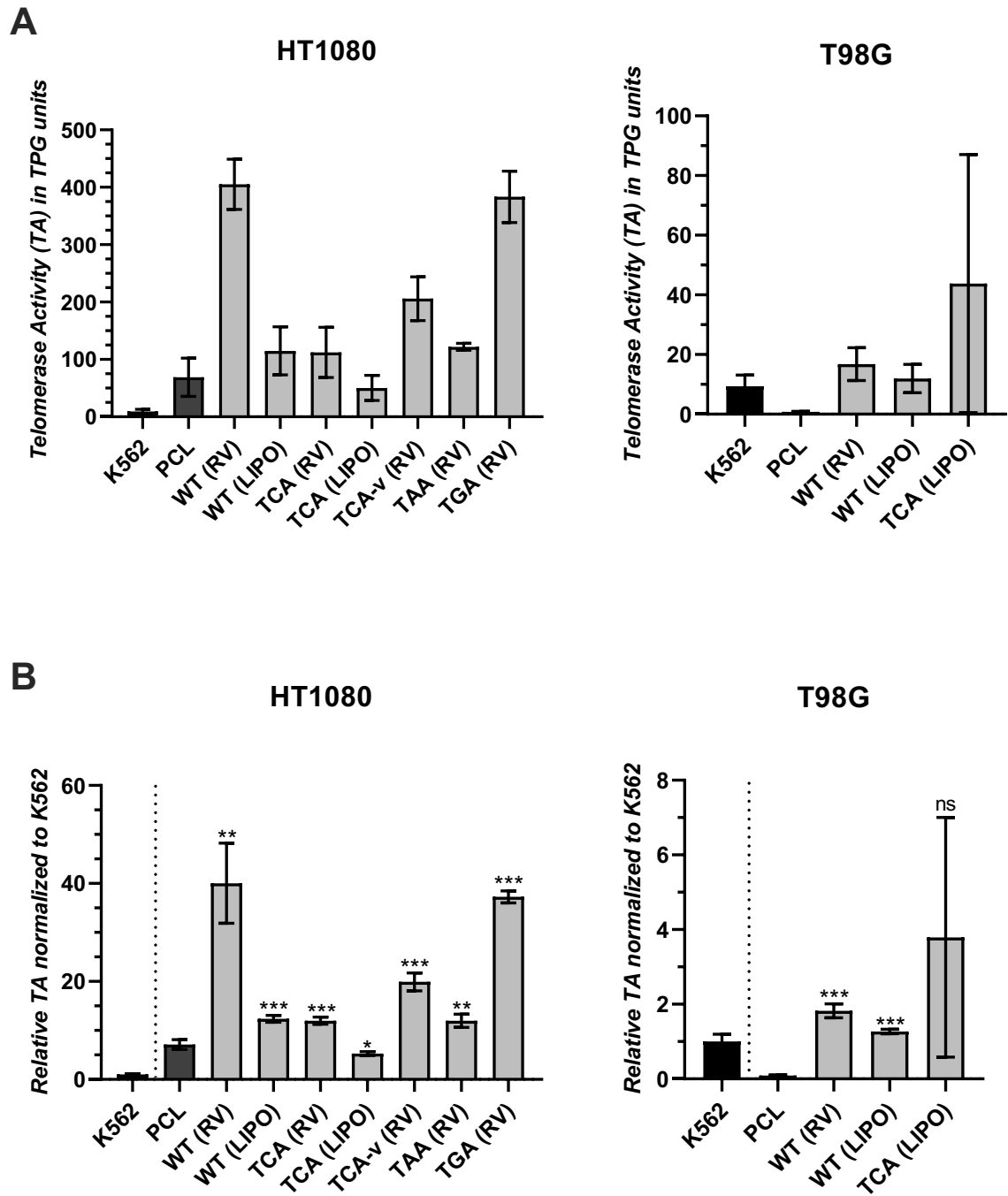


Figure 10 - Telomerase Activity of clones from TA cell lines HT1080 and T98G analysed in two independent qPCR-TRAP experiments. Each experiment included two technical replicates per sample ($n=4$). Error bars show standard deviation. Telomerase activity is shown as TPG units (A). Additionally, TPG ratios were calculated normalized to positive control K562 (B). Significant changes in relative telomerase activity of the overexpression clones were calculated in respect to their parental cell lines using t-Test and Welch's-Test.

4.8 In vitro functionality of ectopic hTERC in complex with endogenous hTERT

In order to add telomeric repeat variants of overexpressed hTERC-mutations to the telomers, the artificial hTERC constructs need to be able to form a functional enzyme complex with the endogenous hTERT subunit of the cells. To confirm the functionality of the ectopic hTERC construct in complex with endogenous hTERT, the extension of artificial telomeric oligonucleotides containing both canonical TTAGGG and non-canonical TCAGGG repeats was assessed. This was done by incubating these oligonucleotides with protein extracts from WT- and TCA- clone variants of the HT1080 and T98G cell lines. In a first step, artificial telomer oligos were elongated by the extracted telomerase complexes of overexpression clones and amplified using a qPCR-TRAP assay. In a second step, TTAGGG and TCAGGG repeats of the amplification products were quantified in an additional qPCR analysis. This preliminary in vitro experiment was based on a combinational assay modified from Glukhov and Cawthon^{3,30}.

As shown in *Figure 11* below, high standard deviation made reliable quantitative interpretation of the results of this preliminary experiment challenging. Nevertheless, changes in TCAGGG to TTAGGG amount were indicated in several WT- and TCA- overexpression clones of cell lines HT1080 and T98G. Regarding HT1080, the relative quantity of the non-canonical TCAGGG repeats tended to decrease in the WT-LIPO variant, when compared to the parental cell line. In contrast, results of the WT-RV variant indicated a slight increase in TCAGGG repeats, similar to the increase of TCAGGG repeats observed in both TCA-variants of cell line HT1080. However, a slight decrease was indicated for the TCA-v-variant. For T98G cells, the results suggested a minor increase of TCAGGG repeats for the WT-LIPO clone and a very prominent increase the non-canonical repeats variant in the TCA-LIPO clone.

In general, reliable quantitative conclusions drawn from the results of this preliminary experiment are very limited, due to high error intervals and lack of repetition.

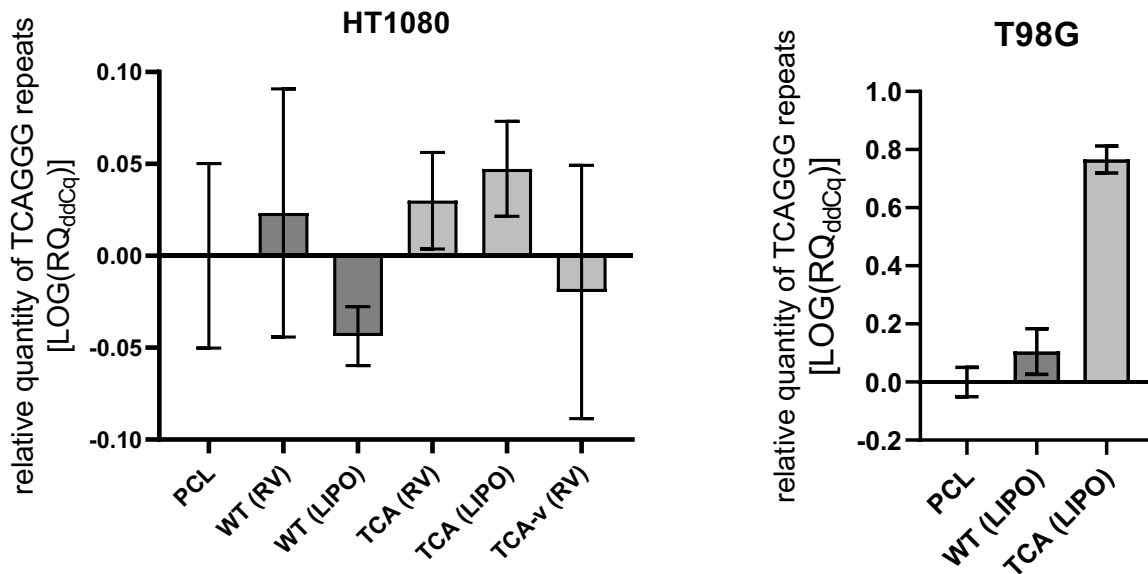


Figure 11 – Relative quantities of TCAGGG repeats added to artificial telomere-primers after qPCR-TRAP reaction. The number of TTAGGG repeats is shown as LOG-transformed quantity relative to the amount of canonical TTAGGG repeats measured, and normalized to the respective parental cell lines using the ddCQ-method. Analysis was performed in a single preliminary experiment with 3 technical repetitions per sample (n=3). The experiment was performed with TCA- and WT-clones of telomerase active cell lines HT1080 and T98G. Error bars show standard deviation. Dark-grey bars show WT-clones, light-grey bars show TCA-clones.

4.9 Integration of WT- and TCA-repeats in telomers of overexpression clones of cell lines HT1080 and T98G

The next step was to analyse whether overexpression of ectopic hTERC variants not only leads to integration of respective variant repeats in vitro but also in situ. Telomeric repeat variants TTAGGG and TCAGGG of WT- and TCA- clones of HT1080 and T98G were quantified using qPCR and normalized to reference gene 36B4 relative to their parental cell line using the ddCQ method. Finally, a combined ratio between the relative quantity of TCAGGG repeats compared to the relative quantity of the canonical TTAGGG repeats was evaluated. A significant increase in TCA-repeats (increase in ratio) was expected to be seen in TCA-overexpression clones, while an increase of TTA-repeats (decrease in ratio) should be visible in WT-overexpression clones.

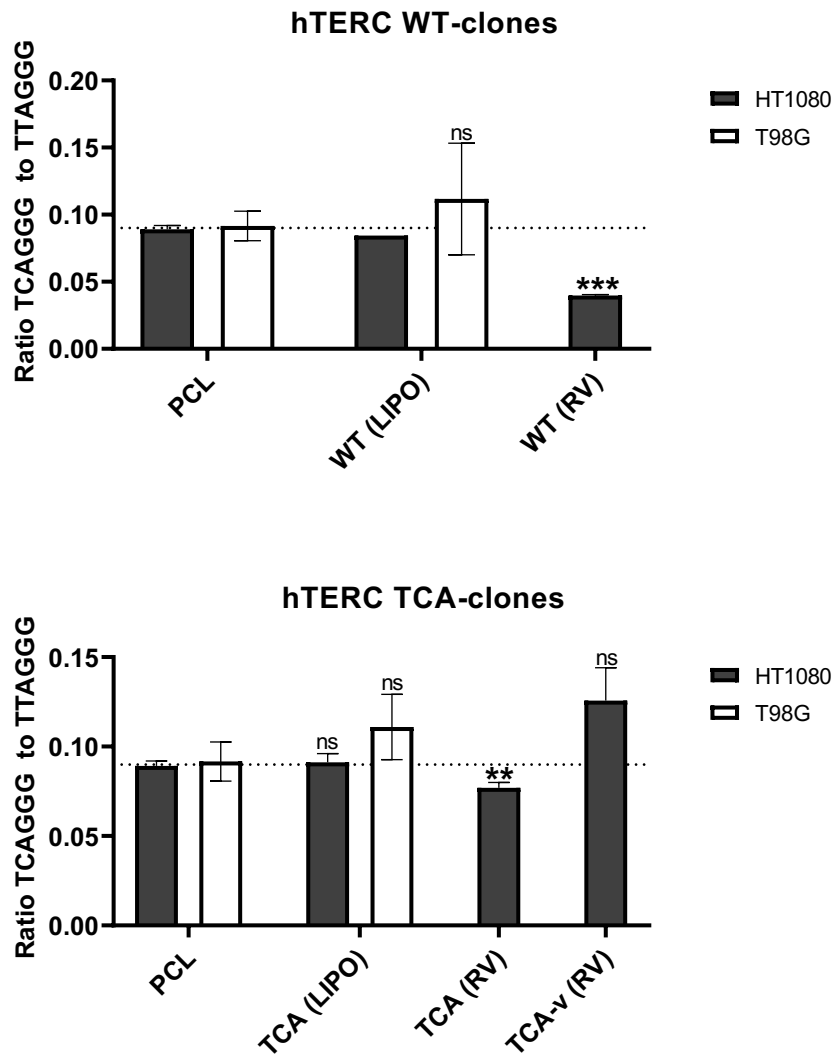


Figure 12 – Mean Ratio of TCAGGG repeats relative to TTAGGG repeats in genomic DNA of WT- and TCA- clones of HT1080 and T98G. Relative quantities (RQ) of both repeat variants were normalized to reference gene 36B4 and the parental cell lines separately using the ddCq-method. Then a ratio of RQ TCATT to RQ TTAGGG was blotted. Error bars show standard deviation. The experiments analysis was performed in up to three independent qPCR-experiments ($n=3$) with 3 technical replicates per sample. Exceptions are HT1080-WT-LIPO ($n=1$) and HT1080-WT-RV ($n=2$). The dotted line represents the threshold value defined by the ratios of the parental cell lines as reference samples. Significant changes in TCAGGG/TTAGGG ratio were calculated between clones and their respective parental cell lines using *t*-test and Welch's test. Gaussian distribution was assumed for HT1080-WT RV data due to lack of third independent qPCR-experiment.

Parental cell lines of HT1080 and T98G showed a very similar level of TCA-TTA ratios at 0.09 ± 0.002 ($n = 3$) and 0.09 ± 0.009 ($n = 3$), setting a combined threshold line at 0.9 to compare the selected clones to (Figure 12).

WT-LIPO variants of both HT1080 and T98G displayed no significant change in ratio when compared to their parental cell lines, indicating no significant increase in the incorporation of the canonical TTAGGG repeat in their telomers. On the other hand, the WT-RV variant of HT1080 displayed a significant decrease in TCAGGG-TTAGGG ratio at 0.04 ± 0.0005 (n=2). Thus, indicating increased incorporation of canonical telomer repeats. No statistical analysis was performed for the WT-LIPO variant of HT1080 due to lack of additional independent qPCR-experiments.

Looking into the TCA clones, none of the analysed samples of both cell lines showed significantly increased incorporation of TCAGGG-repeats. Nevertheless, results may indicate a tendency of elevated TCAGGG repeat incorporation in the TCA-LIPO clone of T98G at 0.11 ± 0.015 (n=3) and the TCA-v clone of HT1080 at 0.13 ± 0.015 (n=3). In contrast, the TCA-RV variant of HT1080 displayed a slight but significant decrease in TCATTT-TTAGGG ratio with 0.8 ± 0.002 (n=3).

In summary, a significant increase in incorporation of canonical repeats was seen in the WT-RV overexpression clone of HT1080 only. No significantly increased TTAGGG-repeat incorporation was detected in any other WT-variants of HT1080 and T98G. Furthermore, no significant increase in incorporation of the non-canonical TCAGGG repeats was detected in any of the four analysed TCA variants.

4.10 Telomere variant fluorescence in situ hybridization

In a final fluorescent in situ hybridisation (FISH) experiment, specific peptide nucleic acid (PNA) probes were hybridized to cells fixed on glass slides binding to the respective telomeric repeat variants TTAGGG and TCAGGG. Probes binding to the canonical TTAGGG repeats emitted green light, probes binding to TCAGGG variants repeats emitted red light, when excited with UV light. Cells with increased abundance of non-canonical TCAGGG repeats incorporated into their telomers were expected to show spots with yellow signal, resulting from colocalization of TTAGGG (green) and TCAGGG repeats (red). Immortalized WI38-VA13-2RA cells were used as positive control due to their upregulated amount of TCAGGG repeats integrated into their telomers. The untreated HT1080 parental cell line was used as negative control.

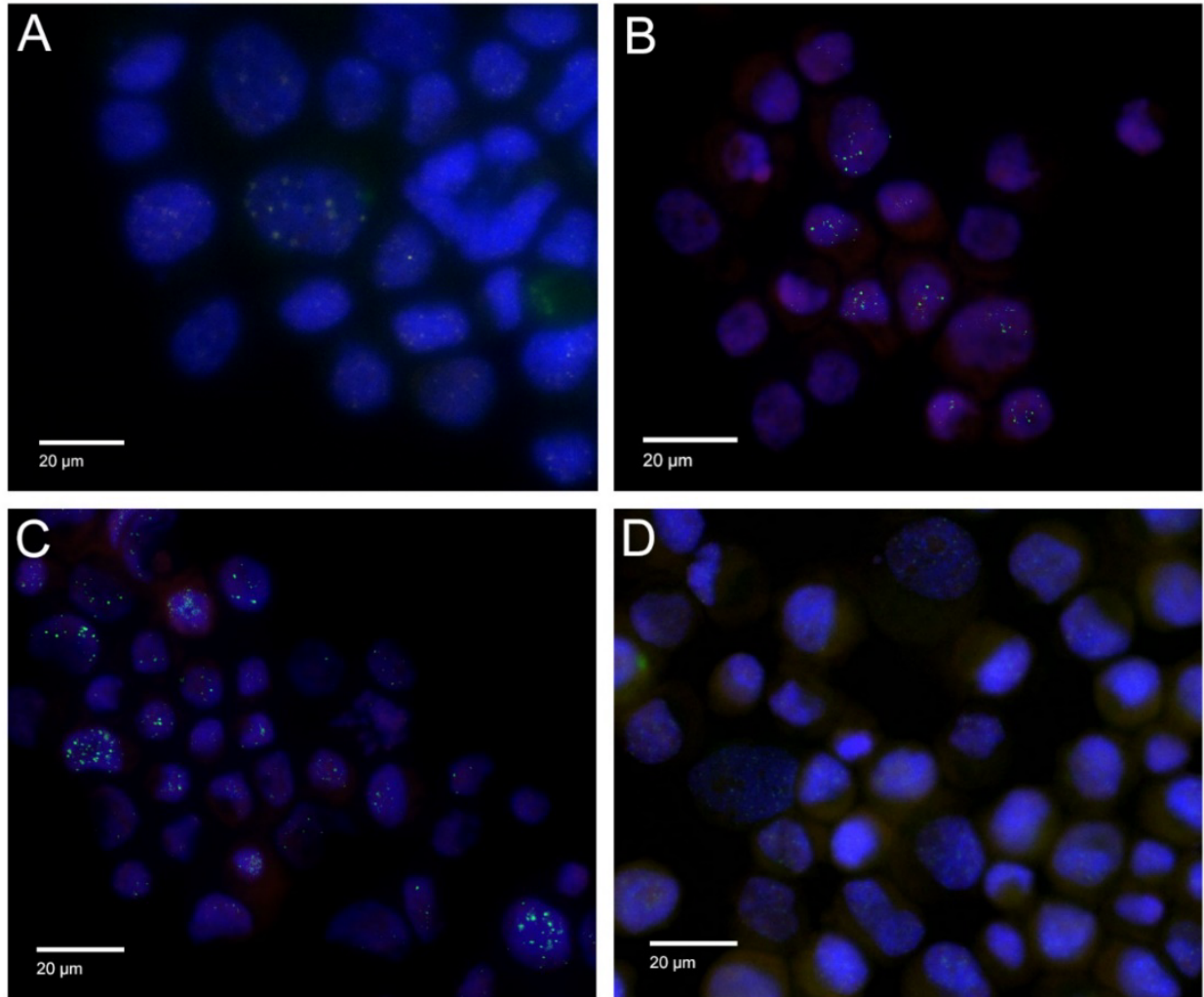


Figure 13 – Overlaid pictures of fluorescent *in situ* hybridization stained with pNA-probes binding to TTAGGG (green) and TCAGGG (red) in WI38-VA13-2RA as positive control (A), HT1080 parental cell line as negative control (B), and hTERT overexpression clones of HT1080-WT-RV (C) and HT1080-TCA-v (D).

When looking at the picture of the positive control, an abundance of yellow signals representing colocalization of TCAGGG and TTAGGG repeats was visible in all cells (Figure 13A). In contrast, untreated HT1080 cells (Figure 13B) showed primarily green signal, indicating a very dominant amount of the canonical repeats localized in their telomeric regions. The HT1080-WT-RV overexpression clones (Figure 13C) showed a prominent green signal, when compared to the negative control. Thus, suggesting an upregulated integration of the overexpressed canonical repeat into the telomers. TCA-v clone variants showed weak green signals and gave no indication of increased TCAGGG repeats incorporation into their telomers (Figure 13D). Hence, the expectation to see increased yellow signal in the TCA-v clone variants was not met.

Overall, results gained from both positive and negative controls suggested that the staining of respective repeats worked as expected. The data gained from this experiment confirmed the hypothesis, that the WT-RV-clone of HT1080 had successfully incorporated the respective overexpressed repeats, whereas the TCA-v clone variant showed no hint of increased amounts TCAGGG repeats incorporated into its telomeric regions.

5 Discussion

The role of telomeric repeat variants abbreviating from the canonical TTAGGG hexamer in cancer cells has been an upcoming research focus in recent years ^{1,23,36}. Certain repeat variant patterns have already been linked to specific cancer types distinguishable by their TMMs ²³. Deepening this knowledge to utilize telomeric repeat patterns as diagnostic or even prognostic markers, could be a promising next step in cancer treatment. Today's gold standard for analysing the highly repetitive structures of telomers is Next Generation Sequencing (NGS), an expensive and highly complex method producing enormous amounts of data. The long-term objective of the underlying project was to establish improved qPCR assays that efficiently quantify both canonical and non-canonical telomeric repeats, thereby offering a cost-effective and less complex alternative to NGS for telomeric repeat quantification. This master thesis was based on publications from Pickett et al 2009 ³¹ and Conomos et al 2012 ¹, demonstrating effects of overexpressing artificial hTERC variants cancer cell lines HT1080 and HeLa.

In an early phase of this project, eight cancer cell lines were screened to identify two cell lines using TA, and two cell lines using ALT to be used in hTERC overexpression experiments. The cell lines were selected based on their endogenous expression patterns of both telomerase subunits hTERT and hTERC. An additional selection factor was the inert resilience to puromycin exposure as selection marker for establishment of stable hTERC overexpression clones later on. Exposure interval and puromycin concentrations used in these cytotoxicity assays were selected to meet thresholds used in similar experiments found in literature ^{1,47-49}. Puromycin concentrations had to be adjusted after a preliminary cytotoxicity assay with Saos2 and U2OS, as the survival rates of cells were unexpectedly low even at the two lowest puromycin concentrations tested (0.5 µg/mL and 1 µg/mL). Adjustment of the puromycin concentrations for the final cytotoxicity assay set up generated more data points at lower concentrations (*Figure 4*), thus giving a clearer picture of the cell survival rates between concentrations 0.1 µg/mL and 1 µg/mL puromycin. All cell lines, with exception of YT-BO, uniformly showed a very low remaining survival rate starting at 1 µg/mL puromycin after 5 days of incubation. Based on this data, it was concluded that 1 µg/mL puromycin was the most reliable concentration as selection threshold for selection of stable hTERC overexpression clones carrying a Puromycin resistance marker (*Figure 4*). In line with literature ³¹, the final incubation time for subsequent clone selection experiments with puromycin was specified at a minimum of 2 weeks to decrease possible survival of cells lacking the target plasmids. To reduce the amount of cell

lines eventually used for the hTERC overexpression experiments, YT-BO was excluded from subsequent experiments based on its elevated resilience to puromycin at 1µg/mL when compared to the other cell lines.

Endogenous hTERC and hTERT expression analysis of the seven remaining cell lines (SW480, SW480-LT, HeLa, HT1080, T98G, U2OS, Saos2) revealed varying expression dynamics of both target genes. ALT cell line Saos2 showed a strong hTERC expression (*Figure 5*) despite neglectable hTERT expression levels, confirming results found in recent literature ^{53,54}. In contrast, U2OS showed hardly any endogenous expression of both telomerase subunits, making it a very interesting candidate for overexpression of ectopic hTERC. Regarding TA cell lines, HT1080 displayed a strong overall expression of both telomeric subunits. HT1080 is a well-established cancer cell line, which has already been used for similar experiments in previous studies, rendering it a useful reference cell line ^{1,23,31}. HeLa also displayed interesting features, showing a very prominent endogenous hTERC expression. Similar to HT1080, HeLa had also already been used in similar experiments by Picket et al 2009 ³¹. However, it was decided that T98G might be a more promising cell line for exogenous hTERC expression, as it displayed the lowest endogenous hTERC expression of all TA cell lines tested (*Figure 5* and *Table 13*). It was hypothesized that this property of T98G might increase potential effects induced by overexpression of exogenous hTERC variants (e.g. incorporation of NCTR into telomeric regions). Consequently, cell lines HT1080, T98G, U2OS and Saos2 were selected for endogenous hTERC overexpression experiments.

Target genes hTERC, PuroR and 3'exohTERC carried on pBABEpuro-U3-hTERC-500 overexpression plasmids were introduced to the four cell lines by Lipofection and Retroviral Transduction in different combinations (*Table 14*). Successful transient hTERC overexpression was shown for Lipofection clones treated with WT- and TCA-hTERC plasmids. hTERC expression increased by a factor of 2.4 and 1.5, respectively (*Figure 5* and *Table 15*). Following these results, it was concluded that the hTERC overexpression plasmids were successfully introduced to the cell lines by Lipofection. Based on transient expression data from HT1080 and T98G, a similar effect was assumed to be present for ALT cell lines U2OS and Saos2 despite missing data. Final confirmation of successful overexpression was awaited from gene expression analysis after selection of stable clones. Since retroviral transduction integrates the target genes directly into the host's genomic DNA ⁵⁵, no transient expression of the target

genes was expected in the clones produced by retroviral infection. Therefore, no transient expression analysis was performed for RV clones.

A total of 22 stable clones were established overexpressing ectopic hTERC variants coding for either the canonical repeat TTAGGG or one of the repeat variants TCAGGG, TAAGGG or TGAGGG (*Table 14*) after selection under by puromycin exposure for up to 12 weeks. As mentioned before, transient expression data of HT1080 and T98G had already shown, that the overexpression plasmids successfully induced transient overexpression of hTERC using lipofection. Additionally, significant increase in total hTERC overexpression was successfully shown in all clones of HT1080, T98G, U2OS and Saos2 introduced to artificial mutant hTERC genes (*Figure 7* and *Table 16*). U2OS overexpression-clones displayed the most prominent relative increase at rates of up to 2140 ± 133.3 , when compared to its parental cell line. However, the magnitude of the effect is partly a consequence of the ddCq normalization method, as the untreated parental cell line used as reference has extremely low endogenous hTERC expression levels.

The hTERC expression levels of HT1080, T98G and Saos2 displayed a different pattern compared to their parental cell lines, which showed a higher endogenous hTERC expression. In the WT-clones, hTERC expression increase ranged between 6- to 23-fold (*Figure 7* and *Table 16*), reflecting a similar magnitude of increase as observed in clones from a previous study that overexpressed WT-hTERC constructs in HT1080 and HeLa cells³¹. Clones of HT1080, T98G and Saos2 treated with mutant hTERC variants (TCA, TCA-v, TAA, TGA), on the other hand, showed smaller increases in hTERC expression ranging between factors 2 to 7. Several studies have demonstrated that certain mutations of hTERC can be linked to diseases in human^{28,56,57}. Hence, the observed reduced increase of hTERC variant expression might indicate that elevated amounts of mutant hTERC potentially result in a fitness disadvantage for the cells.

It should be noted that this study's quantitative expression analysis of the telomeric RNA subunit hTERC was limited to the measurement of total hTERC expression per clone bulk. The qPCR assay used during this study could not differentiate between the endogenous and ectopic hTERC, nor among the various introduced mutant variants. Adapted qPCR assays aiming to recognize the ectopic hTERC constructs (e.g. hTERC variant specific primers) specifically were not available for this master thesis.

Nevertheless, the data of the total hTERC expression suggests that the ectopic hTERT variants were successfully overexpressed in all clones (*Figure 7* and *Table 16*). However, verifying these findings using assays that specifically quantify the ectopic hTERC construct, and differentiate between the individual hTERC constructs, represents a more refined approach for future studies.

To underline the results seen in stable overexpression data, integration of the ectopic hTERC gene into the genomes of the clones was performed. Reliable quantification of the relative hTERC gene copy numbers was not possible in this study due to a yet unsolved issue when using the hTERC qPCR assay on genomic DNA, resulting in high standard deviations (*Figure 9A* and *Table 17*). However, relative copy numbers of peripheral regions of the ectopic hTERC construct, namely PuroR and 3'exohTERC, were successfully quantified (*Figure 9B/C*, *Table 17*). Both regions were found integrated into the genome of all selected cells, which flank the ectopic hTERC sequence on the introduced plasmids (*Figure 8*). Therefore, presence of these genes in the genomic DNA of the selected clones indicates that the ectopic hTERC gene was also integrated into the target cells.

Pickett et al.³¹ reported that overexpression of artificial wild-type hTERC constructs led to a drastic increase of telomerase activity in cancer cell lines HT1080 and HeLa. A comparable effect was found in all but two clones of HT1080 and T98G (HT1080-TCA-LIPO and T98G-TCA-LIPO) in this project (*Figure 10*). hTERC overexpression led to a significant increase of telomerase activity in 8 out of 10 analysed clones. In line with literature³¹, this effect was very prominent in clones expressing the wild-type hTERC variants (*Figure 10*). But also clones expressing mutant hTERC variants TCA, TCA-v, TAA and TGA displayed significantly increased telomerase activity (*Figure 10*). hTERT has long been recognized to be a limiting factor in telomerase activity^{58,59}, whereas hTERC has also been demonstrated to have restrictive effects on telomerase activity^{51,52}. Overall, upregulation of both telomeric subunits has been associated with the progression of carcinogenesis, playing a crucial role in reactivation of telomerase and eventually leading to cell immortalization⁶⁰.

Additionally, functional integration of repeat variants coded by the ectopic hTERC variants was confirmed in an in vitro environment using a combinational RT-2qPCR-TRAP assay adapted from *Glukhov et al. 2021*³ and *Cawthon 2009*³⁰. Regarding a qualitative conclusion, mixed results were observed concerning in vitro functionality of ectopic hTERC in complex with

endogenous hTERT (*Figure 11*). Suggesting, that the expressed ectopic hTERC variants in complex with endogenous hTERT led to incorporation of the respective repeats in HT1080 clone variants WT-LIPO, TCA-LIPO, TCA-RV and T98G clone variant TCA-LIPO. Whereas, an opposed effect was indicated to occur in HT1080 clone variants WT-RV, TCA-v and T98G clone variant WT-LIPO. Overall, results of this preliminary experiment suggest that isolated Telomerase complexes of WT- and TCA-hTERC overexpression clones of HT1080 and T98G incorporate elevated amounts of the respective coded repeats onto artificial telomer-primers (*Figure 11*). Unfortunately, quantitative interpretation of these results was limited by high error intervals in most analysed HT1080 samples. Interestingly, the incorporation effects in T98G samples were much stronger, showing a clear elevation of TCAGGG repeat signal in extracts from TCA-clones. This preliminary data suggests, that the artificial hTERC construct introduced to the cells can form a functional telomerase complex when binding to endogenous hTERT, consequently adding the coded repeats variants to telomeric sequences. Further repetitions and analysis of all clone-variants of HT1080 and T98G presents an interesting option for follow-up studies.

Based on preliminary results, ectopic hTERC can successfully code for the respective repeats in vitro, making integration of the overexpressed telomeric repeat variants in situ seem promising. However, analysis of TTAGGG and TCAGGG repeats in telomers of HT1080 and T98G clones revealed, that only one wild-type clone (HT180 WT-RV) out of seven analysed wild-type and TCA-clones displayed significantly increased numbers of the overexpressed repeat in its telomeric regions (*Figure 12*). The remaining wild-type clones did not show the same effect. Similarly, none of the analysed TCA-clones displayed any significant increase in TCAGGG repeats numbers. Unexpectedly, TCA-RV clone of HT1080 even showed a slight but significant increase in canonical TTAGGG repeats rather than TCAGGG repeats (*Figure 12*).

In summary, only one clone bulk of HT1080 overexpressing wild-type hTERC (WT-RV) significantly increased incorporation of the coded overexpressed variant. In situ telo-FISH staining of TTAGGG (green) and TCAGGG repeats (red) in fixed cells of HT1080-WT-RV and HT1080-TCA-v showed a similar dynamic (*Figure 13*). When compared to positive and negative controls, HT1080-WT-RV showed an increased amount of TTAGGG signal, whereas no increase of TCAGGG signal was found in HT1080-TCA-v clones. As the respective repeat patterns of

TAA- and TGA-clones were not analysed in the course of this study, these clones still need to be looked into.

In this project, DNA samples for gene copy and telomeric repeat analysis were collected after a maximum incubation time of 24 population doublings (pds). In comparison, Pickett et al. described a detectable increase of telomer repeats starting at 50 pds, continuing up to 300 pds in wild-type hTERC overexpression clones of HT1080³¹. Consequently, prolongation of the incubation time prior to sampling of the clones by at least a factor of two might increase the probability of finding increased amounts of variant repeats induced by hTERC overexpression. In addition, all clones analysed here were bulks of heterogenous cells originating from up to over a hundred different clones per culture expressing the introduced target genes in various amounts. This raises the risk that cells with reduced ectopic hTERC gene expression could potentially outgrow and diminish the effects on the clones. To circumvent this problem, application of clonal selection methods as described by Barnes et al 2006⁶¹ and Kondragunta et al 2010⁶² may help to single out cells expressing the introduced genes most efficiently.

Moreover, one might argue that the variant mutations introduced to the template regions of the ectopic hTERC might impair the binding affinity of the guiding RNA to the telomeric sequences. Three of the five introduced hTERC variants (TCA, TAA, TGA) depicted in *Figure 1* carry two nucleotide substitutions, one of which is located on a sequence designated for binding to the correct site of the telomere, as defined by Gavory et al. 2002¹⁵. In contrast, the TCA-v (TCA-variation) hTERC variant carries only one substitution on the coding sequence of the template region, thereby leaving the binding region untouched. It was hypothesized that a single nucleotide substitution at the binding sequence, as seen in TCA-, TAA- and TGA variants potentially impacts the functionality of the artificial hTERC construct. However, when comparing the results from TCA-clones with altered binding region with the TCA-v clones with unaltered binding region, there was no clear indication that the respective change on the binding sequence led to an increased dysfunctionality of the construct (*Figure 11* and *Figure 12*). Furthermore, Conomos et al. 2012¹ and Lee et al. 2014²³ showed successful integration of variant repeats in overexpression clones using hTERC constructs carrying both substitutions.

Another interesting question is whether transient incorporation of variant repeats can be induced in hTERC overexpression clones of ALT cell lines U2OS and Saos2 by activation of

hTERT. Activation of hTERT in ALT cells has already been shown to decrease telomere length heterogeneity by lengthening short telomeres while trimming long telomeres⁶³. It would be very interesting to see if this effect could be reproduced in hTERC overexpression clones of U2OS and Saos2, using the elongation of telomeres by telomerase to incorporate variant repeats.

In conclusion, the combined analysis on mRNA, DNA and protein isolates strongly suggests that in vitro models overexpressing wild-type and mutant hTERC variants were successfully established in HT1080, T98G, U2OS and Saos2. Furthermore, data showed that overexpression of hTERC leads to significant increase of telomerase activity in cell lines using TA as TMM. In addition, overexpression induced significantly increased incorporation of TTAGGG repeats in at least one clone bulk of HT1080 overexpressing ectopic wild-type hTERC. This could be confirmed in both qPCR as well as telo-FISH analysis.

By optimizing incubation time, using clonal selection tools and improving hTERC qPCR assays, similar outcomes might be induced in clone bulks overexpressing TCA-, TAA- and TGA-hTERC mutants as well. Eventually, those hTERC overexpression in vitro models may contribute to establish innovative qPCR assays for quantification of telomeric repeat variants as competitive alternative to NGS, thereby generating a new tool for studies investigating the impact of certain repeat patterns on different cancer types and telopathies.

6 Abbreviations

3'exohTERC	Specific sequence of pBABE-puro-U3-hTERC-500 plasmids
36B4	Housekeeping gene
Absorp.	Absorption
ALT	Alternative Lengthening of Telomers
Amp	Ampicillin
bp	Base Pairs
BSA	Bovine Serum Albumin
Casy TTC, OLS	Automated cell counting device
cDNA	complementary DNA
CHAPS	Detergent for solubilizing proteins
Cy3	Cyanine3
CTRs	Canonical telomere repeats (TTAGGG)
D10	DMEM medium with 10% FCS
DAPI	4',6-Diamidino-2-phenylindol
dCq	Normalization method for qPCR quantification
ddCq	Normalization method for qPCR quantification
DMEM	Dulbecco's Modified Eagle Medium for cell culture
DNA	Deoxyribonucleic Acid
dNTP	Deoxy-Nucleoside-Tri-Phosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylenglycolbis(aminoethylether)-N,N,N
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GC	Gene Copy
GFP	Green Fluorescent Protein
HEK293	Human epithelial kidney cell line
HELA	Human cervical carcinoma cell line
HT1080	Human fibrosarcoma cell line
hTERC	Human Telomerase RNA Component
hTERT	Human Telomerase Reverse Transcriptase
IPTG	Isopropyl- β -D-thiogalactopyranosid

K562	Human myeloid leukaemia cell line
KLD mix	Blend of Kinase, Ligase and DpnI enzymes
LB	Lysogeny Broth
LIPO	Overexpression clones generated using Lipofection
LTR	Long Terminal Repeat
MNP	Growth medium for cell culture
n.d.	not determined
NaP	Sodium Pyruvate
NCTRs	Non-Canonical Telomere Repeats
NEAA	Non-Essential Amino Acids
NGS	Next Generation Sequencing
NTC	Non-Template-Control
P/S	Penicillin-Streptomycin
PBS	Phosphate Buffered Saline
PCL	Parental Cell Line
PCR	Polymerase Chain Reaction
pNA	Peptide Nucleic Acid
POT1	Protection of Telomere 1 (Protein of Shelterin Complex)
PuroR	Puromycin Resistance gene
qPCR	Quantitative Polymerase Chain Reaction
RAP1	Repressor / Activator Protein 1 (Protein of Shelterin Complex)
RNA	Ribonucleic Acid
RNAse A	Ribonuclease A
RNP	Ribonucleoprotein
RPMI-1640	Roswell Park Memorial Institute Medium for cell culture
RQ	Relative Quantity
RT	Room Temperature
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
RT-qPCR-TRAP	TRAP assay using q-PCR for quantification of TRAP products
RV	Overexpression clones generated using Retroviral Infection/Transduction
Saos2	Human Osteosarcoma cell line

SD	Standard Deviation
SDM	Site Directed Mutagenesis
SOC	Growth medium for bacterial cultures
SRB	Sulphorhodamine B
SW480	Human adenocarcinoma cell line
SW480-LT	Human adenocarcinoma cell line
T98G	Human glioblastoma cell line
TA	Telomerase Activity
TAA-variant	hTERC variant coding for TAAGGG telomeric repeats
TBS	Tris Buffered Saline
TCA-v-variant	hTERC variant coding for TCAGGG telomeric repeats. Variation of TCA-variant lacking single nucleotide exchange at telomer binding region.
TCA-variant	hTERC variant coding for TCAGGG telomeric repeats
Tech. reps.	Technical replicates
Telo-FISH	Fluorescence in situ hybridization of telomers
TERC	telomerase RNA component
TERT	telomerase reverse transcriptase
TGA-variant	hTERC variant coding for TGAGGG telomeric repeats
TIN2	TRF1- and TRF2-Interacting Nuclear Protein 2 (Protein of Shelterin Complex)
TMM	Telomer Maintenance Mechanism
TPC	Total Product Generated (unit for telomerase activity measurement)
TPP1	TIN2- and POT1-interacting protein 1 (Protein of Shelterin Complex)
TRAP	Telomeric Repeat Amplification Protocol
TRF1	Telomere Repeat binding Factor 1 (Protein of Shelterin Complex)
TRF2	Telomere Repeat binding Factor 2 (Protein of Shelterin Complex)
Tris	Tris(hydroxymethyl)aminomethane
U2OS	Human osteosarcoma cell line
WI38-VA13-2RA	Human fibroblast cell line
WT-variant	hTERC variant coding for TTAGGG telomeric repeats
X-Gal	5-Brom-4-Chlor-3-Indolyl-beta-D-Galactopyranosid
YTBO	Human glioblastoma cell line

7 Literature

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8 Appendix

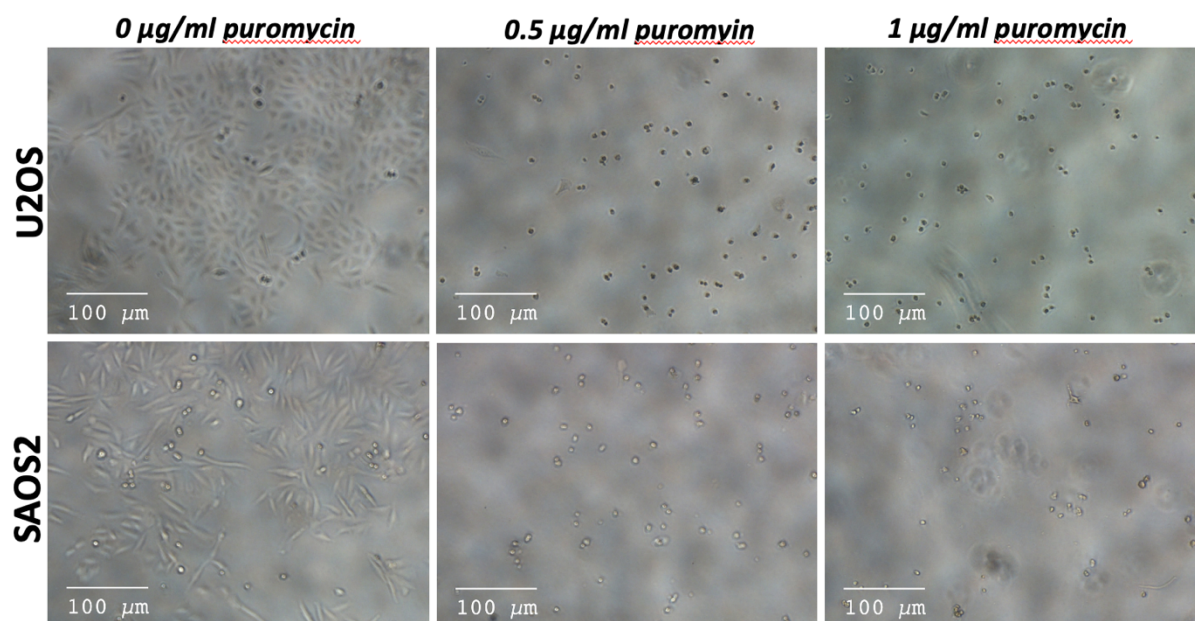
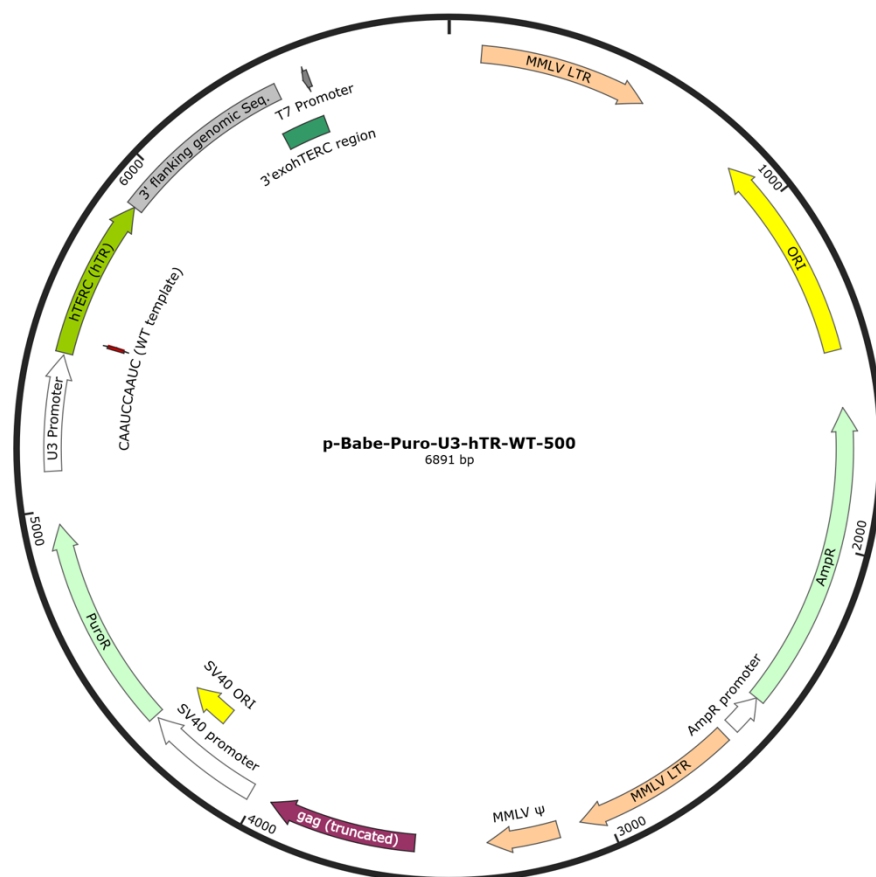


Figure S 1 - Pictures of U2OS and Saos2 cells exposed to 0 µg/mL, 0.5 µg/mL and 1 µg/mL for 5 days. Survival dropped rapidly starting at 0.5 µg/mL puromycin. Phase contrast microscopy mainly showed dead or dying cells in all wells containing puromycin. These pictures shown here, stand as representation for the whole experimental setup. The experiment was aborted due to the low cell survival rate and puromycin concentrations were re-evaluated for a second experimental setup.

A



B



C



D



E

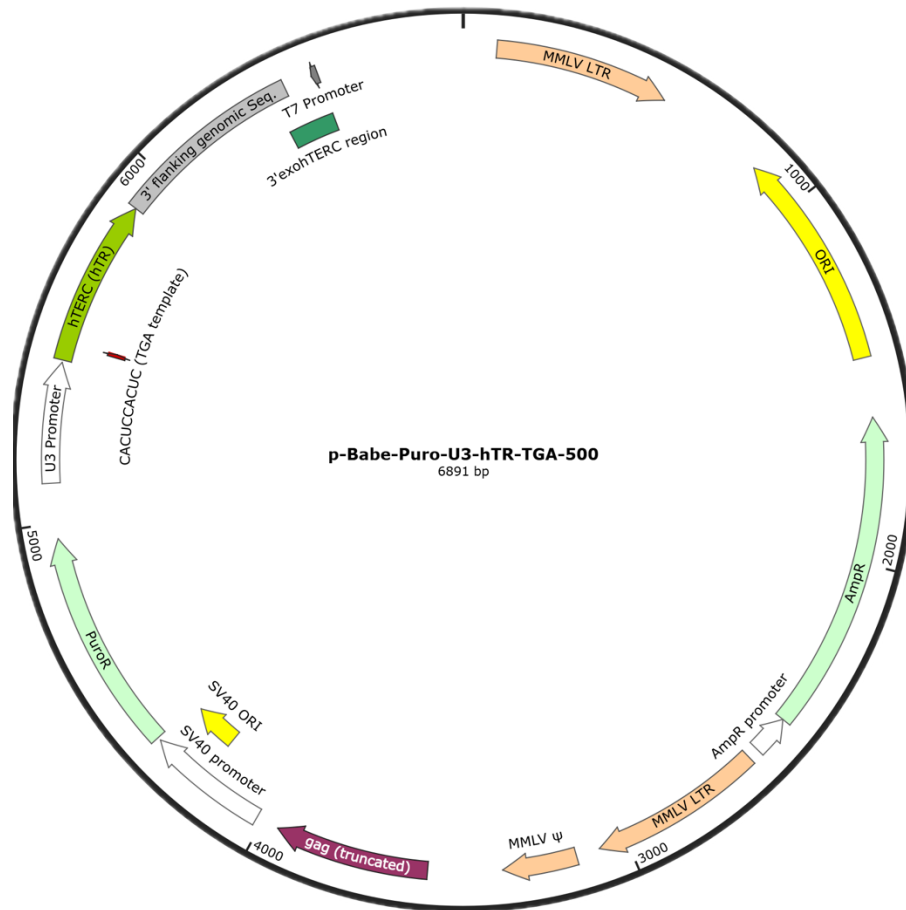


Figure S 2 - Plasmid maps of hTERC (hTR) overexpression plasmids of pBabe-puro-U3-hTR-WT-500^{1,42} (A), pBabe-puro-U3-hTR-TCA-500^{1,42} (B), pBabe-puro-U3-hTR-TCAv-500 (C), pBabe-puro-U3-hTR-TAA-500 (D) and pBabe-puro-U3-hTR-TGA-500 (E). The hTERC gene is shown with CCAUCCAXCU regions as template sequence for the respective telomeric variant repeats. The 3'exohTERC region defines a region located at the transition between the target gene and the pBabe-Puro-backbone. PuroR and AmpR gene are resistant markers for Puromycin and Ampicillin selections.

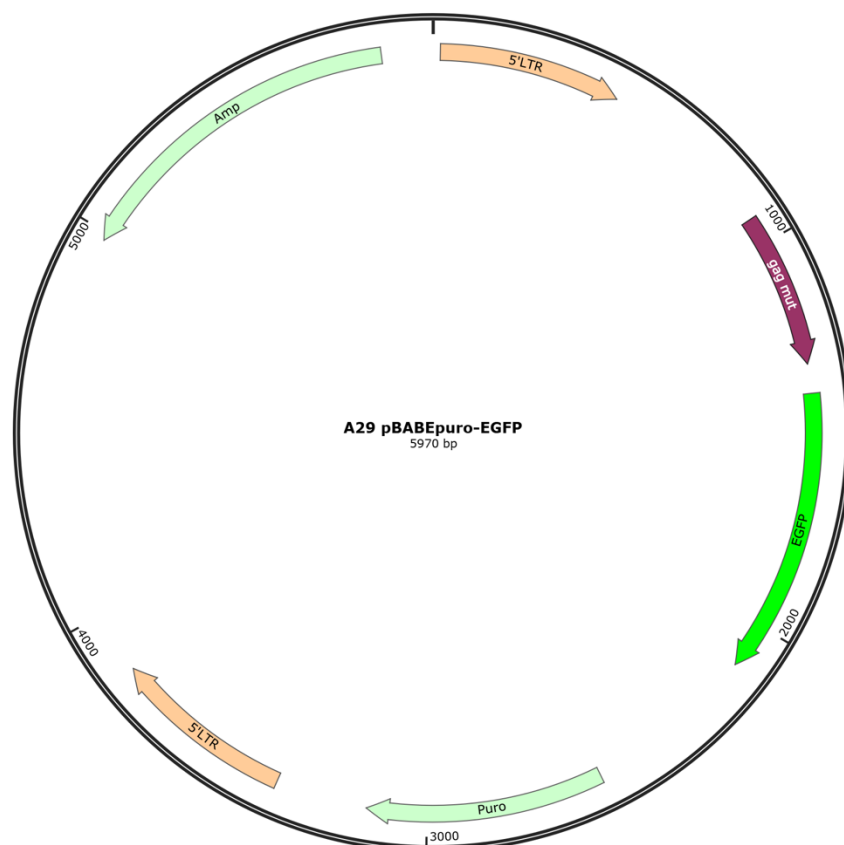


Figure S 3 – Plasmid map of A29 pBABEpuro-EGFP plasmid used for induction of GFP-protein expression in overexpression experiments. The EGFP gene codes for the green fluorescent protein in target cells. This plasmid was kindly provided by Assoc. Prof. Priv.-Doz. Mag. Dr. Michael Grusch.

Table S 1 - Results of the SRB-Assay showing **mean absorption** at wavelength of 570nm of SBR-reagent bound to cellular proteins of viable cells after 5 days of exposure to puromycin. Experiment was performed with six biological replicates per cell line and puromycin concentration (n=6).

Puromycin [µg/mL]	Absorp. mean	Absorp. SD	Absorp. mean	Absorp. SD	Absorp. mean	Absorp. SD
	SW480		SW480-LT		HeLa	
0	1.27	0.147	2.38	0.097	1.07	0.080
0.1	0.75	0.131	1.51	0.241	0.81	0.072
0.2	0.52	0.062	0.85	0.115	0.13	0.021
0.3	0.28	0.043	0.48	0.048	0.03	0.006
0.4	0.21	0.023	0.26	0.052	0.02	0.004
0.5	0.11	0.009	0.13	0.013	0.02	0.001
1	0.05	0.013	0.10	0.009	0.01	0.001
5	0.04	0.003	0.06	0.010	0.02	0.003
	HT1080		T98G		U2OS	
0	1.22	0.186	2.24	0.106	0.55	0.031
0.1	0.88	0.220	2.02	0.193	0.40	0.041

0.2	0.24	0.035	2.04	0.354	0.23	0.044
0.3	0.11	0.020	1.32	0.313	0.15	0.026
0.4	0.06	0.009	0.66	0.234	0.09	0.014
0.5	0.04	0.005	0.33	0.115	0.05	0.007
1	0.03	0.004	0.02	0.002	0.03	0.005
5	0.03	0.008	0.04	0.016	0.04	0.021
	Saos 2		YT-BO			
0	0.34	0.039	0.11	0.022		
0.1	0.20	0.025	0.11	0.026		
0.2	0.12	0.023	0.10	0.020		
0.3	0.08	0.010	0.09	0.022		
0.4	0.06	0.008	0.09	0.018		
0.5	0.05	0.002	0.07	0.011		
1	0.02	0.004	0.04	0.008		
5	0.03	0.008	0.03	0.015		

Table S 2 - Results of the qPCR-analysis of the **endogenous hTERC- and hTERT-expression** in the candidate cell lines prior to transfection and infection. The table shows mean Cq-values (**Cq mean**) with standard deviation (**SD**) and replicate number per sample (**n**). Expression of 36B4 was measured as reference gene.

sample	36B4			hTERT			hTERC		
	Cq mean	SD (Cq)	n	Cq mean	SD (Cq)	n	Cq mean	SD (Cq)	n
HELA	18.97	0.018	2	30.12	0.184	3	19.83	0.011	2
HT1080	19.58	0.005	2	28.73	0.132	3	22.26	0.036	2
SAOS	19.95	0.067	2	38.36	0.000	3	22.39	0.060	2
SW480	18.71	0.077	2	30.18	0.090	3	20.69	0.009	2
SW480-LT	18.56	0.033	2	29.55	0.045	3	21.10	0.009	2
T98G	19.44	0.036	2	30.83	0.094	3	22.78	0.061	2
U2OS	18.70	0.007	2	34.24	0.133	3	32.00	0.001	2
NTC	25.07	0.000	2	39.94	0.000	3	33.93	0.000	2

Table S 3 - Results of the qPCR-analysis of the **transient hTERC-overexpression** after lipofection in HT1080 and T98G. The table shows mean Cq-values (**Cq mean**) with standard deviation (**SD**) and replicate number per sample (**n**). Expression of 36B4 was measured as reference gene.

sample	36B4			hTERC		
	Cq mean	SD (Cq)	n	Cq mean	SD (Cq)	n
HT1080	19.40	0.069	2	22.20	0.03	2
HT1080-TCA	19.62	0.047	2	21.66	0.00	2
HT1080-WT	19.81	0.060	2	21.18	0.07	2
T98G	20.65	0.094	2	24.58	0.00	2
T98G-TCA	19.89	0.003	2	23.06	0.06	2
T98G-WT	23.02	0.014	2	25.98	0.07	2
NTC	0.00	0.000	2	33.88	0.00	2

Table S 4 - Results of the qPCR-analysis of stable **hTERC-overexpression** analysis showing mean Cq-value and standard deviation (SD) of hTERC and 36B4 for each run and sample. Technical replications (**tech. reps.**) of each run and replicate numbers per run (**n**) are shown.

Sample	tech. reps.	36B4 expression			hTERC expression		
		Cq mean	SD (CT)	n	Cq mean	SD (CT)	n
HT1080	Run1	21.07	0.04	2	24.05	0.04	2
	Run2	20.71	0.02	2	24.59	0.12	2
HT1080-TAA RV	Run1	20.43	0.02	2	22.95	0.11	2
	Run2	20.56	0.02	2	23.51	0.10	2
HT1080-TCA Lipo	Run1	19.87	0.01	2	21.21	0.01	2
	Run2	19.90	0.06	2	22.14	0.05	2
HT1080-TCA RV1	Run1	19.47	0.02	2	21.07	0.01	2
	Run2	19.43	0.01	2	21.90	0.04	2
HT1080-TCA-v RV	Run1	20.58	0.05	2	22.86	0.07	2
	Run2	20.82	0.03	2	23.48	0.15	2
HT1080-TGA RV	Run1	19.74	0.01	2	22.02	0.02	2
	Run2	19.89	0.01	2	22.71	0.02	2
HT1080-WT Lipo	Run1	19.29	0.03	2	19.56	0.01	2
	Run2	19.22	0.00	2	20.07	0.02	2
HT1080-WT RV	Run1	20.66	0.01	2	20.13	0.02	2
	Run2	20.78	0.03	2	20.71	0.07	2
SAOS2	Run1	21.29	0.04	2	24.32	0.04	2
	Run2	21.43	0.08	2	25.07	0.08	2
SAOS2-TCA Lipo	Run1	22.93	0.09	2	21.91	0.08	2
	Run2	22.82	0.00	2	21.76	0.05	2
SAOS2-WT Lipo	Run1	21.02	0.03	2	19.17	0.03	2
	Run2	21.10	0.02	2	19.25	0.03	2
T98G	Run1	21.00	0.04	2	24.49	0.03	2
	Run2	20.73	0.19	2	25.11	0.07	2
T98G-TCA Lipo	Run1	21.98	0.04	2	23.58	0.02	2
	Run2	21.90	0.04	2	24.73	0.03	2
T98G-WT Lipo	Run1	22.30	0.04	2	22.70	0.01	2
	Run2	22.18	0.15	2	23.53	0.03	2
T98G-WT RV1	Run1	21.80	0.03	2	22.28	0.00	2
	Run2	21.67	0.04	2	23.09	0.00	2
U2OS	Run1	20.25	0.01	2	32.75	0.21	2
	Run2	20.16	0.03	2	33.91	0.37	2
U2OS-TAA RV	Run1	20.40	0.03	2	22.18	0.13	2
	Run2	20.55	0.00	2	23.11	0.00	2
U2OS-TCA Lipo	Run2	19.39	0.03	2	22.00	0.18	2
	Run1	19.32	0.00	2	20.32	0.00	2
U2OS-TCA RV	Run1	20.17	0.00	2	21.63	0.07	2
	Run2	20.31	0.04	2	22.75	0.10	2
U2OS-TCA-v RV	Run1	20.27	0.02	2	21.39	0.03	2
	Run2	20.31	0.02	2	22.26	0.09	2
U2OS-TGA RV	Run1	20.53	0.08	2	20.85	0.00	2

	Run2	20.66	0.04	2	21.65	0.03	2
U2OS-WT Lipo	Run1	24.20	0.05	2	23.73	0.02	2
	Run2	24.28	0.01	2	24.87	0.00	2
U2OS-WT RV	Run1	24.06	0.00	2	25.87	0.07	2
	Run2	24.29	0.03	2	26.64	0.02	2
NTC	Run1	36.43	1.00	2	33.24	0.13	2
	Run2	n.d.		2	n.d.		2

Table S 5 - Results of the qPCR-analysis of **target gene copies** showing integration of ectopic hTERC gene by quantification of genes hTERC, PuroR and 3'exohTERC. Table shows mean Cq-values (**Cq mean**) and standard deviation (**SD**) of hTERC, PuroR, 3'exohTERC and reference gene 36B4 for each run and sample. Technical replications (**tech. reps.**) of each run and replicate numbers per run (**n**) are shown.

Sample	tech. reps.	36B4			hTERC		
		Cq mean	SD (Cq)	n	Cq mean	SD (Cq)	n
HT1080	Run1	26.26	0.03	2	24.91	0.29	2
	Run2	26.40	0.30	2	26.12	0.03	2
	Run3	26.52	0.37	2	25.17	1.13	2
HT1080-TAA RV	Run1	26.59	0.11	2	26.44	0.03	2
	Run2						
	Run3						
HT1080-TCA Lipo	Run1	28.24	0.18	2	26.87	0.04	2
	Run2	26.17	0.21	2	26.16	0.04	2
	Run3	26.41	0.28	2	26.07	0.22	2
HT1080-TCA RV	Run1	27.30	0.13	2	26.19	1.11	2
	Run2	26.50	0.25	2	26.30	0.02	2
	Run3	27.22	0.00	1	24.77	0.02	2
HT1080-TCA-v RV	Run1	27.61	0.16	2	25.54	0.07	2
	Run2						
	Run3						
HT1080-TGA RV	Run1	27.26	0.22	2	25.45	0.06	2
	Run2						
	Run3						
HT1080-WT Lipo	Run1	27.50	0.09	2	25.83	0.60	2
	Run2	25.89	0.40	2	25.39	0.07	2
	Run3	26.07	0.42	2	25.60	0.02	2
HT1080-WT RV	Run1	23.60	0.17	2	20.81	0.06	2
	Run2	25.66	0.26	2	25.31	0.02	2
	Run3	25.82	0.38	2	25.44	0.01	2
SAOS2	Run1	27.36	0.01	2	28.47	3.05	2
	Run2	26.68	0.27	2	26.43	0.02	2
	Run3						
SAOS2-TCA Lipo	Run1	30.04	0.16	2	27.25	0.01	2

	Run2	28.43	0.27	2	25.66	0.07	2
	Run3						
SAOS2-WT Lipo	Run1	27.44	0.25	2	24.23	0.94	2
	Run2	27.02	0.28	2	23.88	0.79	2
	Run3						
T98G	Run1	25.27	0.25	2	25.28	0.04	2
	Run2	27.33	0.19	2	27.34	0.06	2
	Run3	27.55	0.23	2	27.52	0.12	2
T98G-TCA Lipo	Run1	28.19	0.03	2	26.82	0.10	2
	Run2	26.60	0.28	2	26.56	0.07	2
	Run3	26.90	0.29	2	27.17	0.03	2
T98G-WT Lipo	Run1	26.91	0.11	2	26.39	0.07	2
	Run2	27.26	0.20	2	25.80	0.79	2
	Run3	27.52	0.28	2	27.30	0.06	2
T98G-WT RV	Run1	27.43	0.03	2	26.29	0.03	2
	Run2	27.36	0.25	2	26.03	0.01	2
	Run3	27.59	0.26	2	27.80	0.27	2
U2OS	Run1	27.30	0.18	2	29.16	0.08	2
	Run2	25.83	0.27	2	27.68	0.12	2
	Run3						
U2OS-TAA RV	Run1	27.66	0.21	2	27.16	0.20	2
	Run2						
	Run3						
U2OS-TCA Lipo	Run1	28.37	0.35	2	28.01	0.03	2
	Run2	24.34	0.32	2	22.47	0.02	2
	Run3						
U2OS-TCA RV	Run1	27.56	0.14	2	28.12	0.91	2
	Run2	27.12	0.14	2	27.17	0.05	2
	Run3						
U2OS-TCA RV	Run1	27.17	0.25	2	28.93	0.01	2
	Run2						
	Run3						
U2OS-TCA-v RV	Run1	26.98	0.17	2	27.83	0.08	2
	Run2						
	Run3						
U2OS-TGA RV	Run1	27.83	0.19	2	27.47	0.01	2
	Run2						
	Run3						
U2OS-WT Lipo	Run1	28.80	0.29	2	26.89	0.17	2
	Run2	26.57	0.26	2	25.01	0.54	2
	Run3						
U2OS-WT RV	Run1	24.67	0.40	2	22.69	0.03	2
	Run2	25.89	0.27	2	26.58	0.07	2
	Run3						

Sample	tech. reps.	PuroR			3'exohTERC		
		Cq mean	SD (Cq)	n	Cq mean	SD (Cq)	n
HT1080	Run1	38.65	0.00	1	0.00	0.00	2
	Run2	35.34	0.39	2	0.00	0.00	2
	Run3	35.08	0.40	2			
HT1080-TAA RV	Run1	27.17	0.01	2	27.27	0.02	2
	Run2						
	Run3						
HT1080-TCA Lipo	Run1	29.00	0.01	2	28.10	0.06	2
	Run2	26.55	0.09	2	25.60	0.02	2
	Run3	26.66	0.06	2	25.82	0.03	2
HT1080-TCA RV	Run1	28.41	0.06	2	26.64	0.22	2
	Run2	27.03	0.18	2	25.91	0.05	2
	Run3	27.40	0.02	2	26.02	0.17	2
HT1080-TCA-v RV	Run1	28.60	0.02	2	26.99	0.09	2
	Run2						
	Run3						
HT1080-TGA RV	Run1	28.13	0.00	2	27.13	0.02	2
	Run2						
	Run3						
HT1080-WT Lipo	Run1	27.34	0.07	2	26.57	0.12	2
	Run2	25.68	0.16	2	24.83	0.21	2
	Run3	25.99	0.07	2	25.13	0.08	2
HT1080-WT RV	Run1	24.26	0.03	2	22.97	0.04	2
	Run2	26.38	0.11	2	24.49	0.20	2
	Run3	26.49	0.13	2	24.82	0.06	2
SAOS2	Run1	28.76	0.07	2	35.85	0.15	2
	Run2	27.58	0.03	2	34.33	0.29	2
	Run3						
SAOS2-TCA Lipo	Run1	27.46	0.08	2	25.44	0.17	2
	Run2	26.04	0.04	2	23.99	0.06	2
	Run3						
SAOS2-WT Lipo	Run1	24.79	0.02	2	23.14	0.13	2
	Run2	24.52	0.03	2	22.87	0.13	2
	Run3						
T98G	Run1	37.02	1.41	2	n.d.	n.d.	2
	Run2	35.40	0.00	1	0.00	0.00	2
	Run3	0.00	0.00	2	0.00	0.00	2
T98G-TCA Lipo	Run1	29.69	0.08	2	29.03	0.01	2
	Run2	27.68	0.17	2	27.23	0.12	2
	Run3	28.01	0.05	2	27.49	0.12	2
T98G-WT Lipo	Run1	27.64	0.13	2	26.35	0.04	2
	Run2	27.24	0.28	2	26.18	0.09	2
	Run3	27.77	0.12	2	26.38	0.01	2
T98G-WT RV	Run1	29.96	0.14	2	29.03	0.01	2
	Run2	29.13	0.29	2	28.20	0.04	2

U2OS	Run3	29.69	0.07	2	28.38	0.12	2
	Run1	36.41	0.59	2	n.d.	n.d.	2
	Run2	38.84	0.00	2	38.22	0.00	2
	Run3						
U2OS-TAA RV	Run1	28.61	0.17	2	26.16	0.06	2
	Run2						
	Run3						
U2OS-TCA Lipo	Run1	27.79	0.06	2	26.29	0.03	2
	Run2	23.31	0.00	1	22.79	0.03	2
	Run3						
U2OS-TCA RV	Run1	28.21	0.02	2	26.20	0.06	2
	Run2	27.99	0.03	2	25.80	0.06	2
	Run3						
U2OS-TCA RV	Run1	28.33	0.16	2	26.55	0.03	2
	Run2						
	Run3						
U2OS-TCA-v RV	Run1	27.54	0.04	2	25.30	0.07	2
	Run2						
	Run3						
U2OS-TGA RV	Run1	28.80	0.02	2	26.09	0.02	2
	Run2						
	Run3						
U2OS-WT Lipo	Run1	27.76	0.13	2	26.73	0.08	2
	Run2	25.22	0.13	2	24.79	0.31	2
	Run3						
U2OS-WT RV	Run1	25.33	0.05	2	23.65	0.07	2
	Run2	27.11	0.11	2	25.09	0.06	2
	Run3						

Table S 6 - Results of the qPCR-TRAP analysis of **the telomerase activity** of HT1080 and T98G clone bulks. Table shows mean Cq-values (**Cq mean**) and standard deviation (**SD**) of each sample. Technical replications (**tech. reps.**) of each run and replicate numbers per run (**n**) are shown. K562 was used as reference cell line.

Sample	tech. reps.	Cq mean	SD (Cq)	n
HT1080	Run1	23.68	0.12	2
	Run2	22.41	0.05	2
	Run3			
HT1080-TAA RV	Run1	22.08	0.02	2
	Run2	22.09	0.08	2
	Run3	21.59	0.00	2
HT1080-TCA Lipo	Run1	24.04	0.07	2
	Run2	22.90	0.01	2
	Run3			
HT1080-TCA-v RV	Run1	21.57	0.05	2
	Run2	21.13	0.07	2
	Run3	21.13	0.01	2
HT1080-TGA RV	Run1	20.58	0.01	2
	Run2	20.30	0.05	2
	Run3	20.52	0.09	2
HT1080-WT Lipo	Run1	22.70	0.01	2
	Run2	21.78	0.10	2
	Run3			
HT1080-WT RV	Run1	20.23	0.00	2
	Run2	20.49	0.07	2
	Run3	20.25	0.05	2
H-TCA RV	Run1	22.78	0.07	2
	Run2	21.79	0.08	2
	Run3			

K562	Run1	26.38	0.34	2
	Run2	25.38	0.11	2
	Run3			
T98G	Run1	29.59	0.12	2
	Run2	29.13	0.09	2
	Run3			
T98G-TCA Lipo	Run1	26.29	0.01	2
	Run2	22.68	0.11	2
	Run3			
T98G-WT Lipo	Run1	26.03	0.05	2
	Run2	25.00	0.05	2
	Run3			
T98G-WT RV	Run1	25.41	0.13	2
	Run2	24.58	0.06	2
	Run3			

Table S 7 - Results of the 2qPCR-TRAP-analysis of the *in vitro* functionality of ectopic hTERC. Table shows mean Cq-values (**Cq mean**) and standard deviation (**SD**) of each sample. Technical replications (**tech. reps.**) of each run and replicate numbers per run (**n**) are shown.

Sample	Tel		Tel-C		n
	Cq mean	SD (Cq)	Cq mean	SD (Cq)	
HT1080	18.37	0.03	24.56	0.16	3
HT1080-TCA Lipo	18.66	0.07	24.69	0.04	3
HT1080-TCA RV	19.67	0.14	26.00	0.09	3
HT1080-TCA-v RV	18.82	0.15	25.20	0.11	3
HT1080-WT Lipo	18.47	0.03	24.89	0.03	3
HT1080-WT RV	19.26	0.08	25.25	0.06	3
K562	21.77	0.09	27.15	0.09	3
T98G	19.65	0.19	26.32	0.31	3
T98G-TCA Lipo	25.16	0.04	29.38	0.14	3
T98G-WT Lipo	20.30	0.22	26.62	0.16	3
CHAPS-Control	34.90	0.24	30.47	0.07	3
NTC	31.86	0.18	29.21	0.05	2

Table S 8 - Results of the qPCR-analysis of *T*TAGGG- and *T*CAGGG-repeats in the telomers of stable HT1080- and T98G-clone bulks with 36B4 as reference gene. Table shows mean Cq-values (**Cq mean**) and standard deviation (**SD**) of each sample. Technical replications (**tech. reps.**) of each run and replicate numbers per run (**n**) are shown. 36B4 was used as reference gene.

Sample	tech. repl.	36B4		Tel		Tel-C		n
		Cq mean	SD (Cq)	Cq mean	SD (Cq)	Cq mean	SD (Cq)	
HT1080	Run1	25.39	0.09	19.58	0.06	24.61	0.06	3
	Run2	25.49	0.12	19.33	0.05	24.29	0.04	3
	Run3	25.51	0.05	19.26	0.03	24.35	0.06	3
HT1080-TCA Lipo	Run1	25.25	0.02	19.09	0.05	24.03	0.03	3
	Run2	25.35	0.03	18.86	0.01	23.61	0.01	3
	Run3	25.39	0.08	18.87	0.12	23.82	0.01	3
HT1080-TCA RV	Run1	25.45	0.08	19.12	0.01	24.45	0.04	3
	Run2	25.51	0.05	18.91	0.04	24.09	0.03	3
	Run3	25.31	0.04	18.73	0.02	23.89	0.07	3
HT1080-TCA-v RV	Run1	25.92	0.08	19.63	0.16	24.32	0.05	3
	Run2	26.11	0.02	19.66	0.09	23.83	0.08	3
	Run3	26.10	0.02	19.34	0.13	23.54	0.04	3
HT1080-WT Lipo	Run1	25.01	0.06	18.52	0.13	23.42	0.09	3
	Run2							
	Run3							
HT1080-WT RV	Run1	24.92	0.06	16.00	0.07	21.96	0.07	3
	Run2	24.76	0.04	15.88	0.03	21.87	0.05	3
	Run3							
T98G	Run1	25.35	0.05	20.20	0.45	25.13	0.00	3
	Run2	25.52	0.09	19.89	0.20	25.33	0.69	3
	Run3	25.17	0.09	19.47	0.02	24.26	0.07	3
T98G-TCA Lipo	Run1	26.05	0.34	20.27	0.21	25.01	0.18	3
	Run2	25.54	0.11	20.46	0.28	24.80	0.17	3

	Run3	25.88	0.14	20.03	0.02	25.01	0.12	3
T98G-WT Lipo	Run1	26.23	0.03	20.68	0.14	25.97	0.12	3
	Run2	26.21	0.00	20.38	0.04	25.36	0.11	3
	Run3	25.93	0.14	21.17	0.06	25.37	0.06	3
NTC	Run1	n.d.	n.d.	34.85	0.22	29.39	0.14	2
	Run2	33.44	0.26	34.18	0.13	28.52	0.06	2
	Run3	32.79	0.00	33.85	0.06	28.37	0.04	2