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INSTITUT LADY DAVIS DE RECHERCHES MÉDICALES / LADY DAVIS INSTITUTE FOR MEDICAL RESEARCH

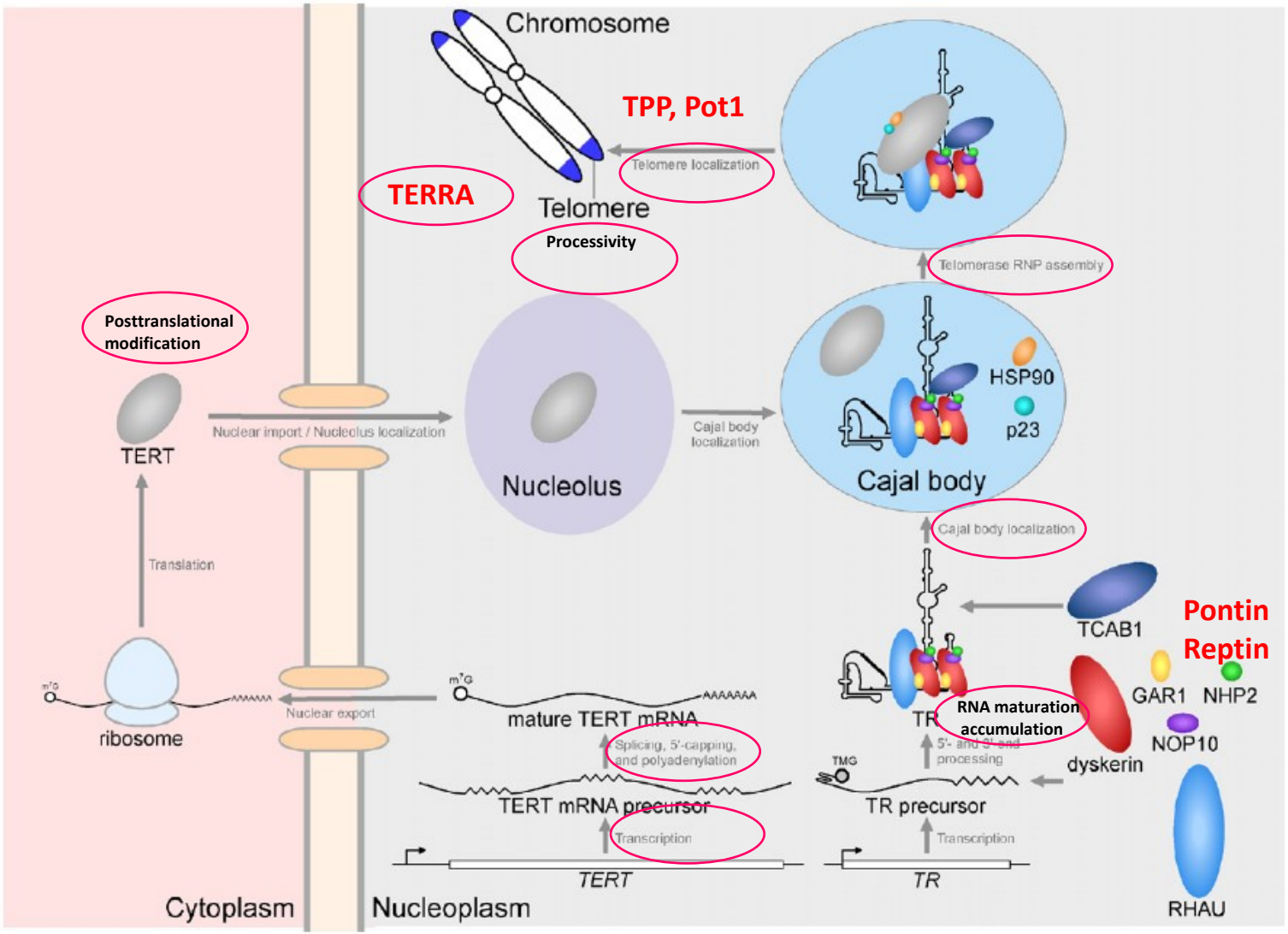
Centre Bloomfield de recherche sur le vieillissement

The Bloomfield Centre for Research in Aging

Cancer and Aging: Two faces of the same coin

(4) Telomerase and Telomere Regulation

Telomerase and Telomere Regulation



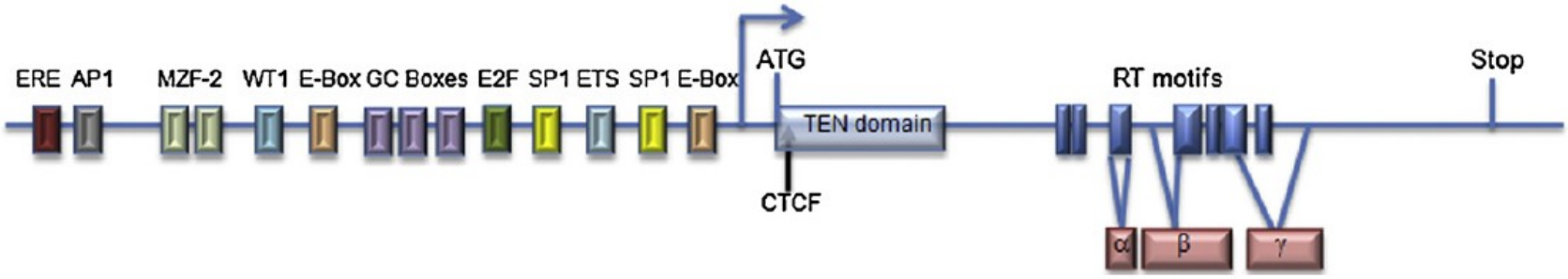
Podlevsky, J. and Chen, J.-J.L. 2012. It all comes together at the ends: Telomerase structure, function, and biogenesis. Mutation Research 730, 3-11.

Transcriptional Regulation of hTERT

<u>Positive regulators</u>: c-myc, Sp1, estrogen receptor, NF-KB (mTERT)

<u>Negative regulators</u>: WT1 (Wilms tumor 1 suppressor), MZF-2 (myeloid-specific zinc finger protein implicated in cell cycle progression), p53

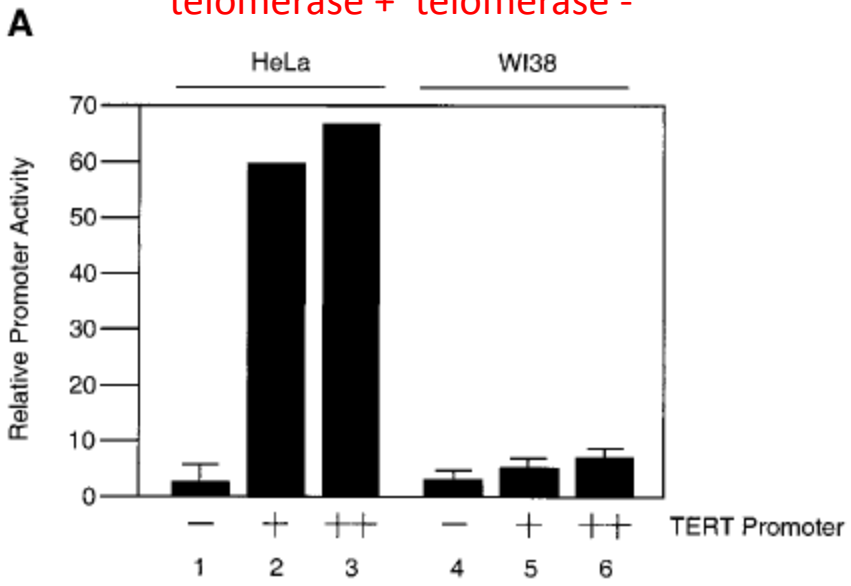
Chromatin and epigenetic regulation of the hTERT gene

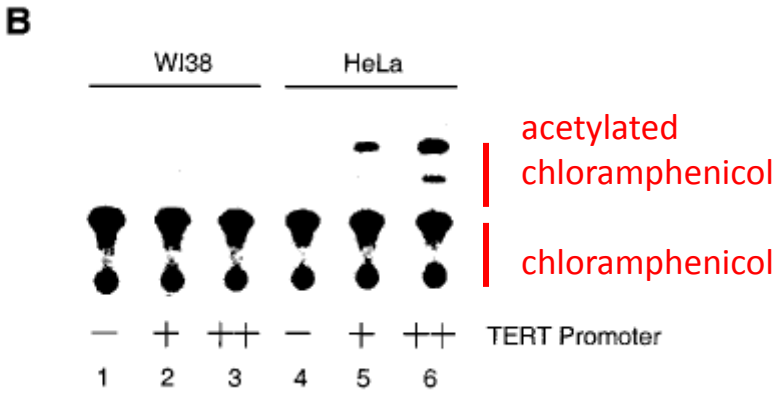


Cifuentes-Rojas, C. and Shippen, D.E. 2012. Telomerase regulation. Mutation Research 730, 20-27.

hTERT promoter inactive in telomerase-negative cells

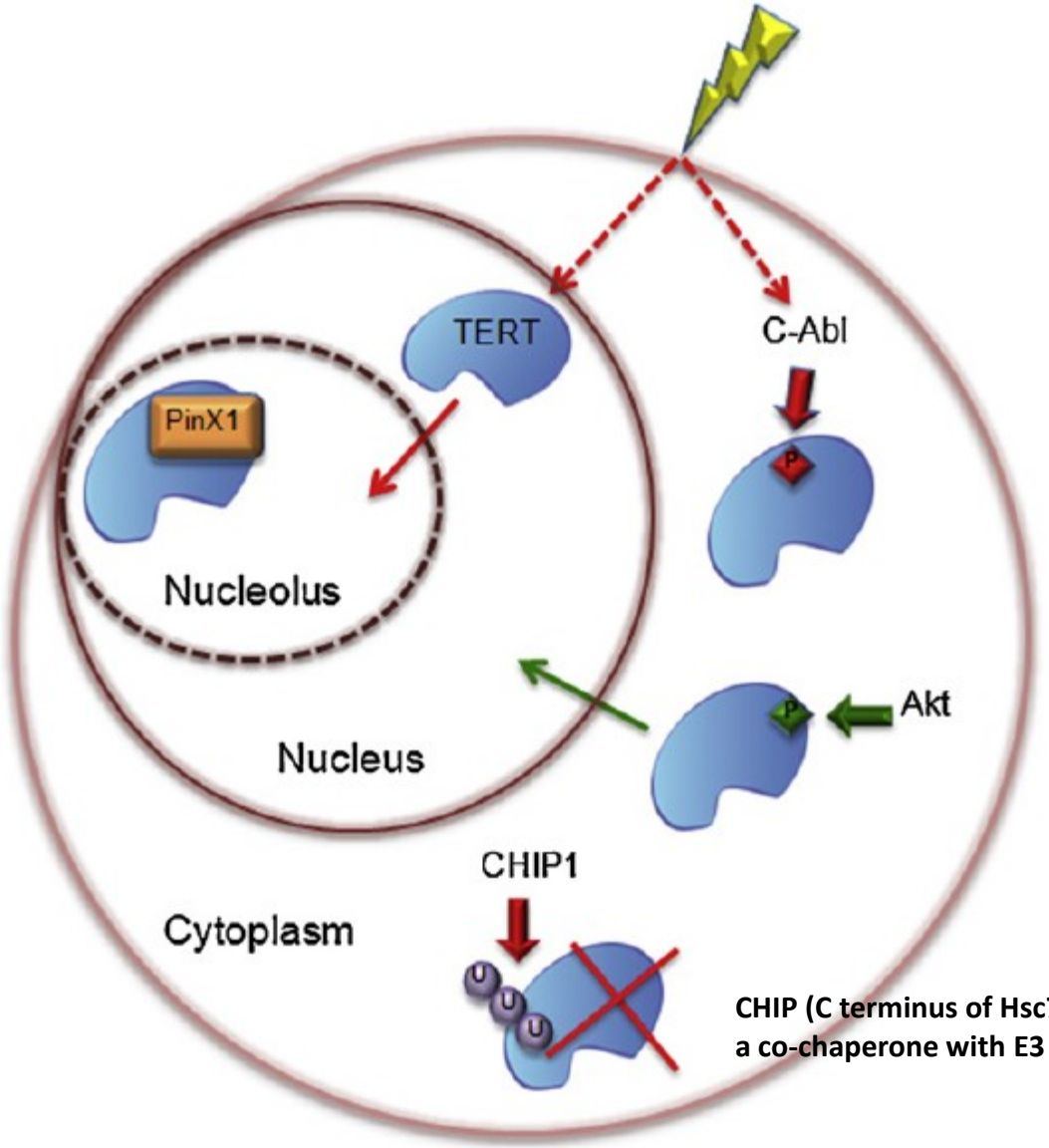
telomerase + telomerase -





Oh, S. et al. 1999. BBRC 263, 361-365.

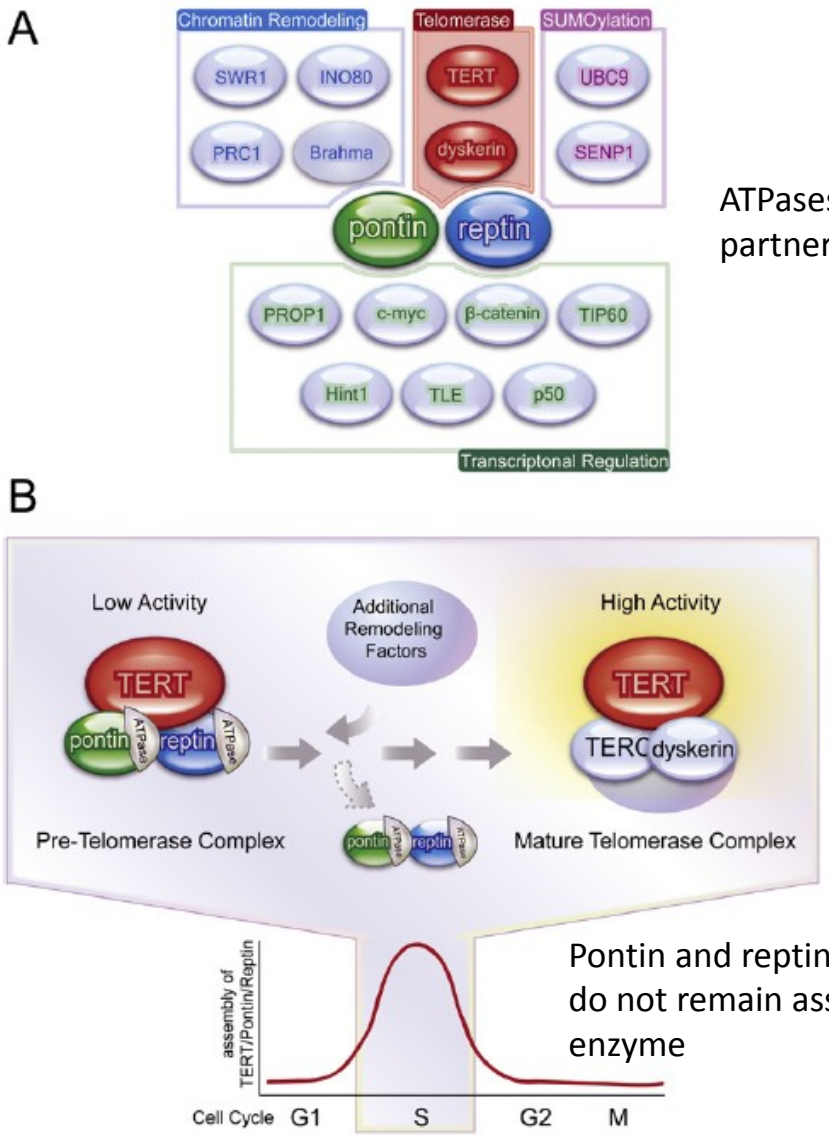
Post-Translational Regulation of TERT



CHIP (C terminus of Hsc70-interacting protein), a co-chaperone with E3 ubiquitin ligase

Cifuentes-Rojas, C. and Shippen, D.E. 2012. Telomerase regulation. Mutation Research 730, 20-27.

Roles for pontin and reptin in telomerase RNP accumulation and assembly

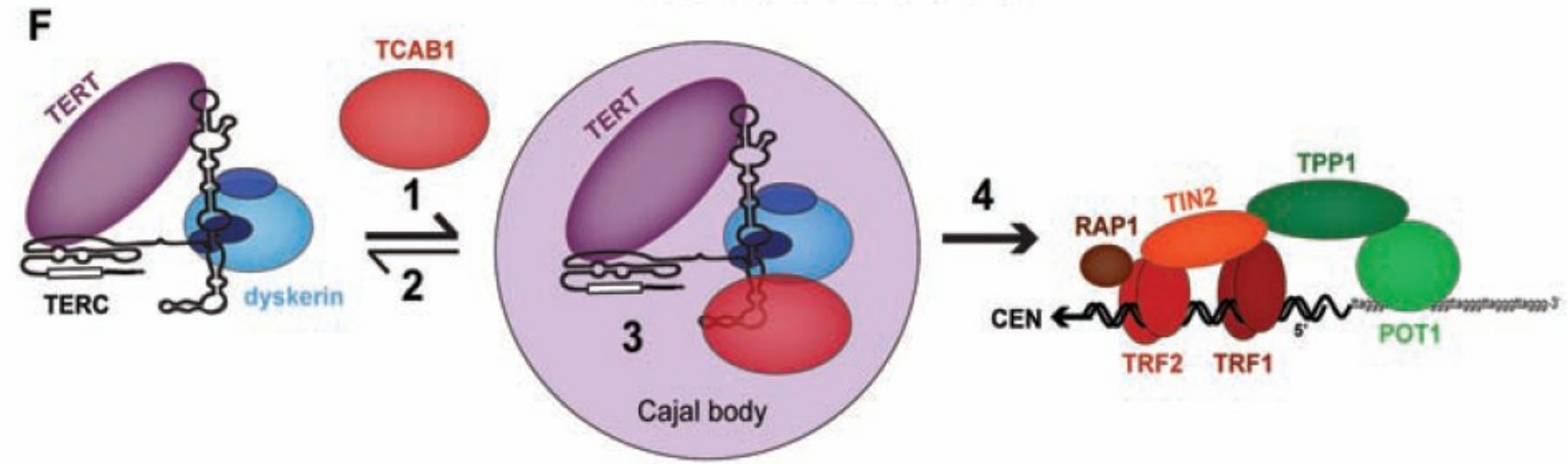


ATPases with multiple binding partners and functions

Pontin and reptin, like NAF1, aid in RNP assembly but do not remain associated with the mature telomerase enzyme

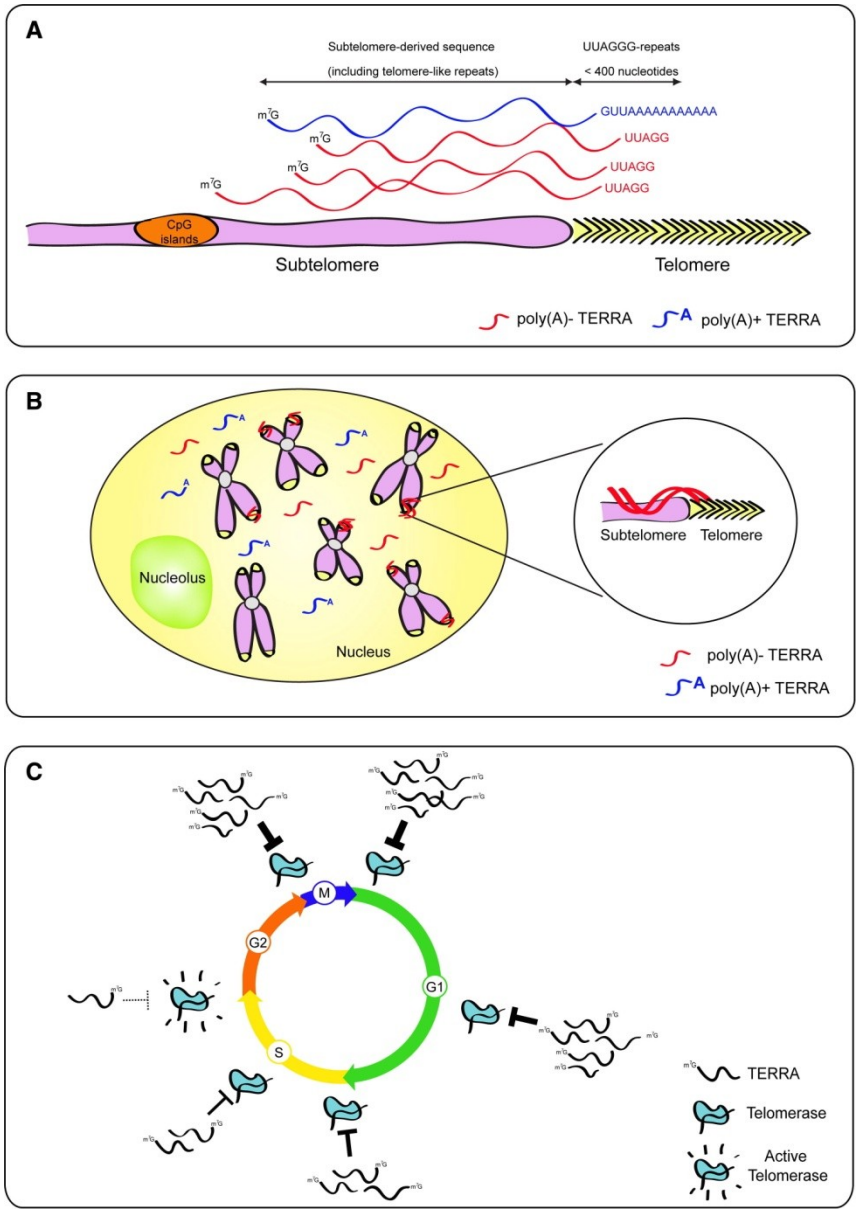
Baek, S.H. 2008. When ATPases pontin and reptin met telomerase. Cell 14, 459-450.

TCAB1: driving telomerase to Cajal bodies and telomeres



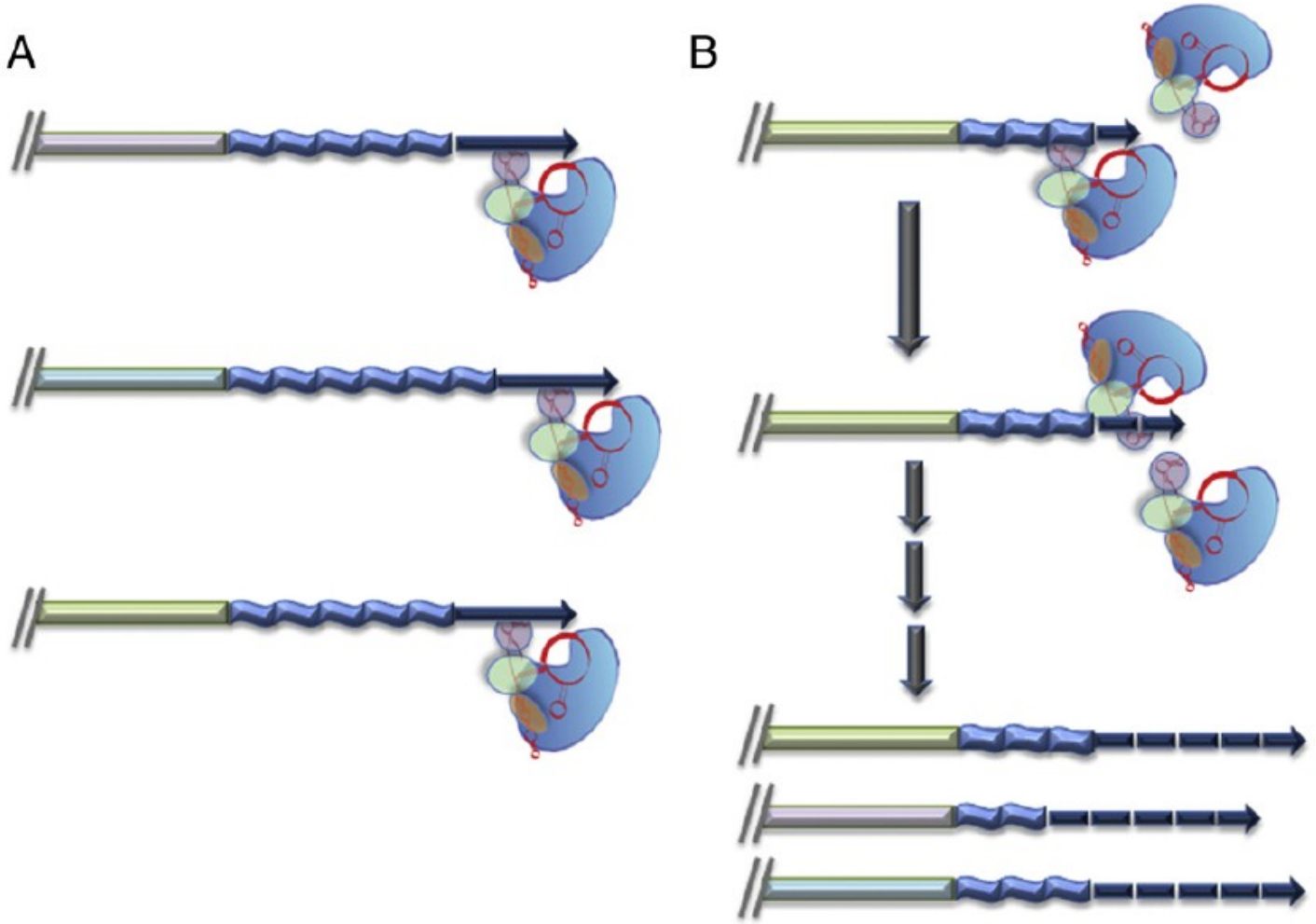
Venteicher, A.S. et al. 2009. A human telomerase holoenzyme protein required for cajal body localization and telomere synthesis. Science 323, 644-648.

TERRA, telomeric RNA



Feuerhahn, S. et al. 2010. FEBS Lett. TERRA biogenesis, turnover and implications for function. 584, 3812-3818.

Telomerase processivity and telomere length regulation

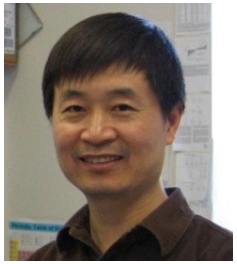


Cifuentes-Rojas, C. and Shippen, D.E. 2012. Telomerase regulation. Mutation Research 730, 20-27.

Unique characteristics of telomerase and telomeres as potential anti-cancer targets

Yasmin D'Souza

Contribution of telomerase processivity to telomere function

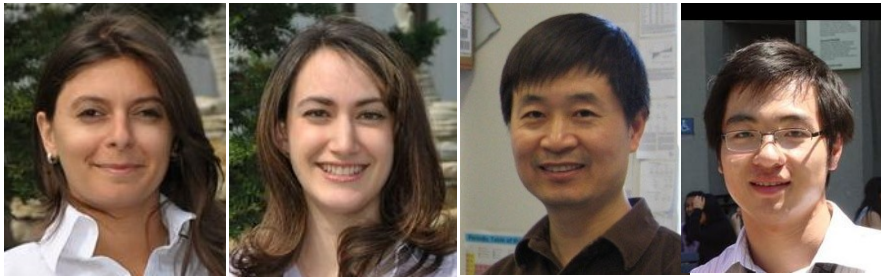


Shusen Zhu and Sanjida Khondacker

Human and mouse telomerase spliced variants

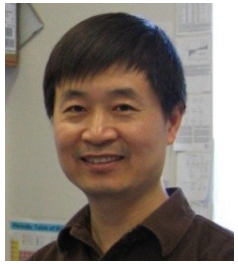
Josephine Chu

Role of unique 'insertion in fingers motif' in telomere function



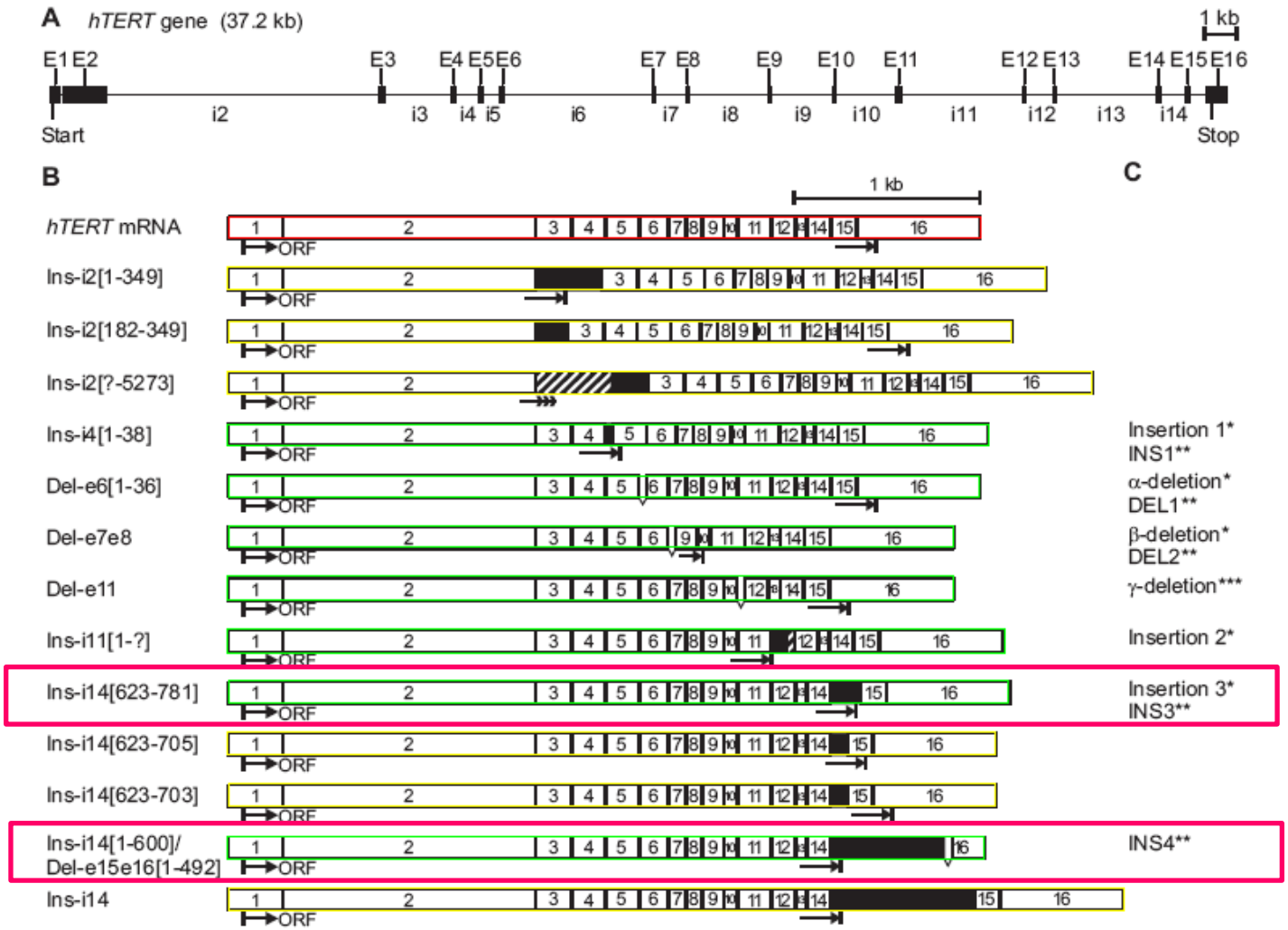
May Shawi, Johanna Mancini, Shusen Zhu, Ricky Kwan

Human and mouse telomerase-associated proteins



The Role of Alternatively-Spliced INS3 and INS4 hTERT mRNAs in Telomerase Function

Genomic organization and alternatively spliced sites of the *hTERT* gene



From: Saeboe-Larssen, S., E. Fossberg, and G. Gaudernack. 2006. *BMC Mol. Biol*. 7 :26-35.

Role of alternatively spliced TERT mRNA variants?

The potential for complex splicing patterns may reflect a specific aspect of telomerase regulation in proliferation, differentiation and apoptosis

Notably, some splice variants are expressed in primary and cancer cells that lack detectable telomerase activity

In human development, the specific expression of hTERT splice variants that are predicted to encode catalytically-defective telomerases, correlates with telomere shortening and suggests that these transcripts may have important physiological roles

Ectopic expression of the α-deletion variant that lacks conserved catalytic residues results in dominant-negative inhibition of telomerase, telomere shortening, and cell death

Spliced TERT variants have also been reported in rice, chicken, mouse and rat

Arabidopsis POT1A reported to interact with an N-terminal variant of telomerase

The G-quadruplex ligand A549 induces telomerase downregulation by modulating hTERT alternative splicing

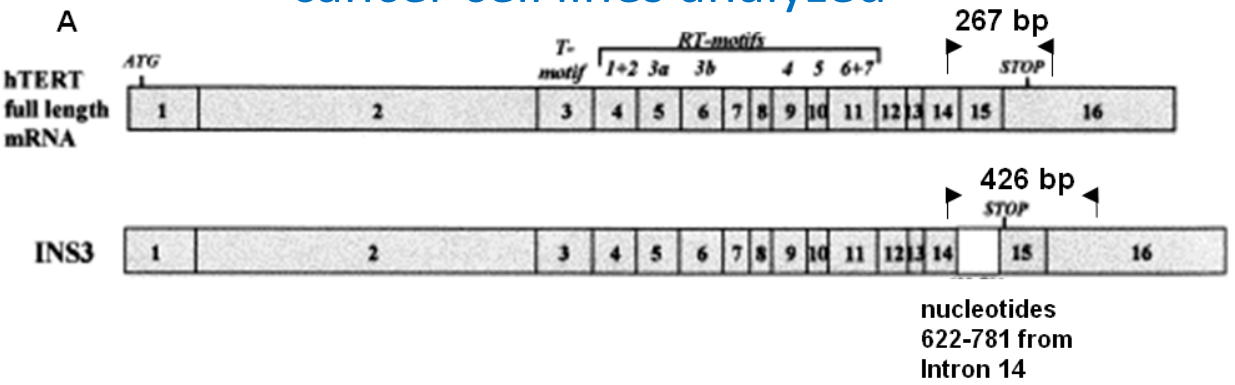
Reviewed in Sykorova, E. and Fajkus, J. 2009. Biol. Cell 101, 375-392)

Alternative splicing of TERT in other organisms

Organism	Alternate splicing	Tissue	Reference
R. norvegicus	Yes	Brain, various adult tissues	Kaneko et al. (2006)
M. musculus	No/yes[a]/yes[b]	Brain/embryonic fibroblast cell line/embryo, brain	Kaneko et al. (2006); Kunicka, Z. and Sýkorová, E., unpublished work[a]/GenBank®[b]
Mesocricetus auratus	Yes[c]	Normal and induced cancer cells (cheek pouch model), other cancer cell lines (unspecified)	Guo et al. (2001)[c]
Canis familiaris	No[d]/yes[e]	Cell lines/mammary gland tumour and normal adjacent tissues	Nasir et al. (2004); Angelopoulou et al. (2008)
Xenopus laevis	No[f]/yes[g]	Developmental stages/whole body	Kuramoto et al. (2001)/Genbank®[e]
Oryzias latipes	Yes	Ovary/testis, developmental stages	Pfennig et al. (2008)
D. rerio	Yes/yes[h]	Not experimental/muscle	Lau et al. (2008)/GenBank®[f]
G. gallus	Yes[i]	Lymphocytes, splenic cells	Hrdlickova et al. (2006)
		Embryo, liver, cell lines	Chang and Delany (2006)
A. thaliana	Yes	Inflorescences, leaves	Rossignol et al. (2007)
O. sativa	Yes	Callus, flag leaf, immature embryo, cell suspension culture	Heller-Uszynska et al. (2002)
Z. mays	Yes	Seedlings	Sykorova et al. (2006b)
Doryanthes excelsa	Yes	Root tips	Sykorova et al. (2006b)
Muscari armeniacum	Yes	Root tips, callus	Sykorova, E. and Fajkus, J. unpublished work, GenBank®
Hosta rectiflora	Yes	Seedlings	Sykorova et al. (2006b)
Ornithogalum virens	Yes	Root tips	Sykorova et al. (2006b)
Iris tectorum	Yes	Root tips	Sykorova et al. (2006b)

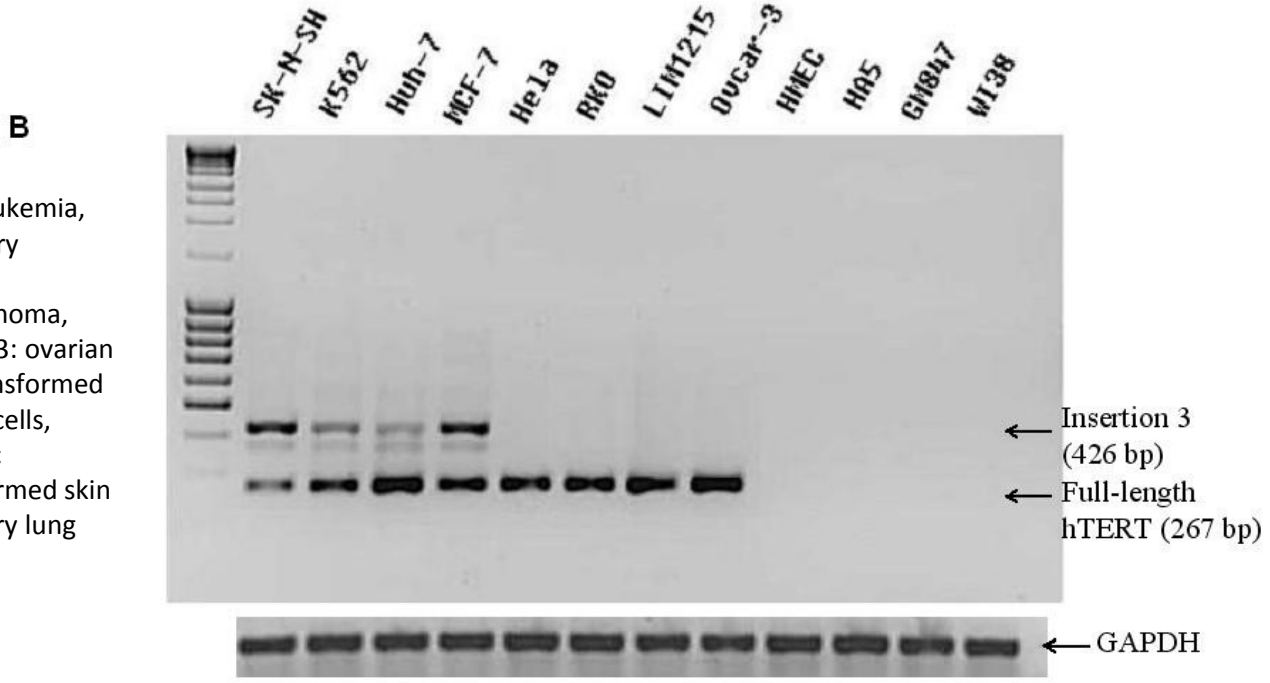
Sykorova, E. and Fajkus, J. 2009. Biol. Cell 101, 375-392

Expression of INS3 spliced hTERT mRNA in some telomerase positive cancer cell lines analyzed



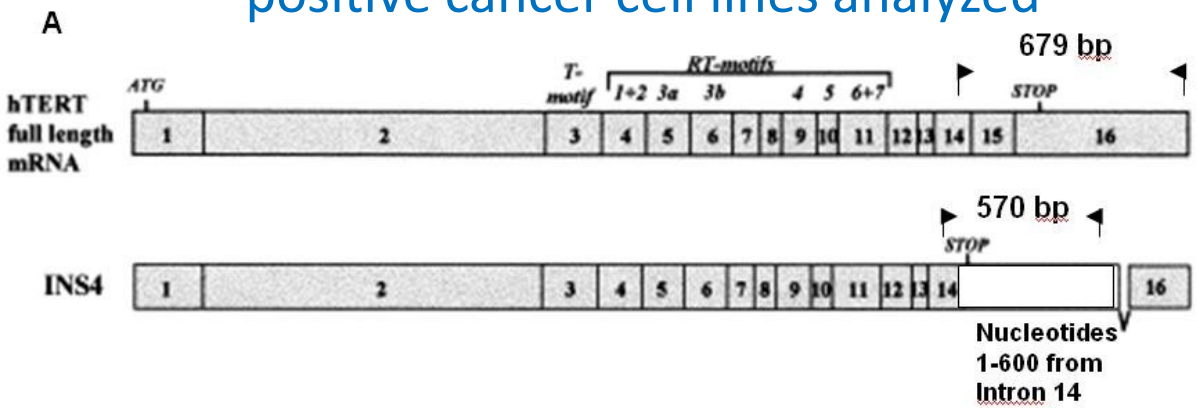
WT 1051-NA GMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQ LSRKLPGTTLTALEAAANPALPSDFKTILD*-1132

INS3 1051-NA AEENISVVTPAVLGSGQ**PEMEPPRRP**SGVGSFPVSPGRGVGLGL*



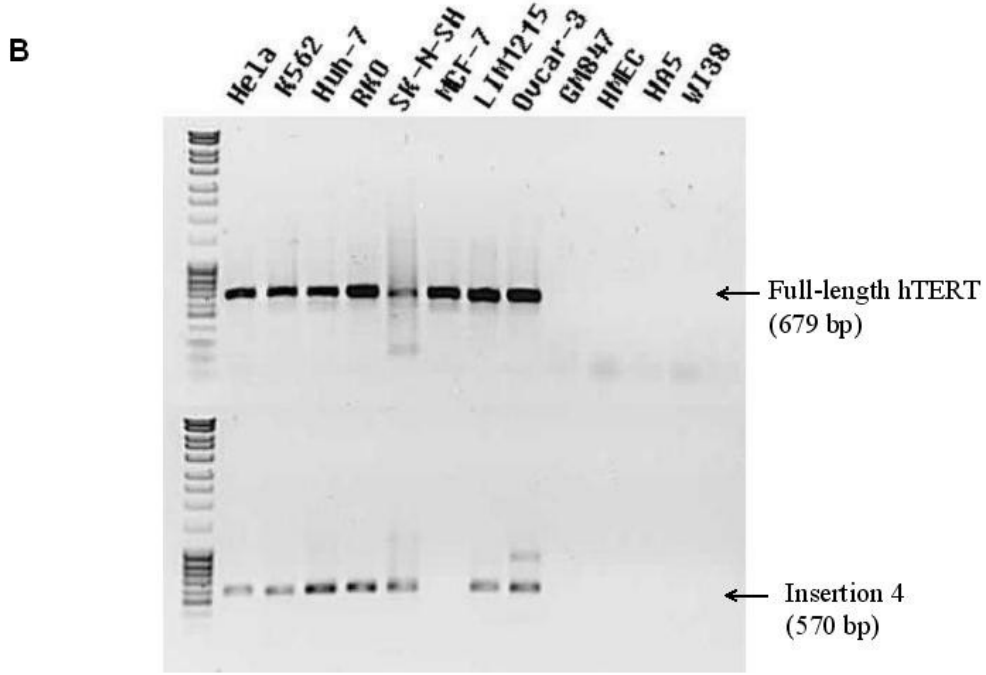
SK-N-SH: neuroblastoma, K562: leukemia, Huh-7: hepatoma, MCF-7:mammary adenocarcinoma, HeLa: cervical adenocarcinoma, RKO: colon carcinoma, LIM1215: colon carcinoma, Ovcar-3: ovarian adenocarcinoma, HMEC: SV40 transformed dermal microvascular endothelial cells, HA5: SV40 transformed embryonic kidney cells, GM847: SV40 transformed skin fibroblasts (ALT cells), WI38:primary lung fibroblasts.

Expression of INS4 spliced hTERT mRNA in most telomerase-positive cancer cell lines analyzed

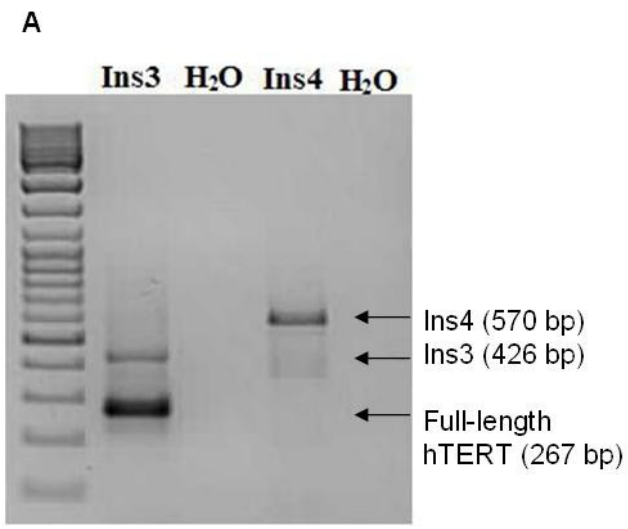


WT 1051-NA GMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSR
KLPGTTLTALEAAANPALPSDFKTILD*-1132

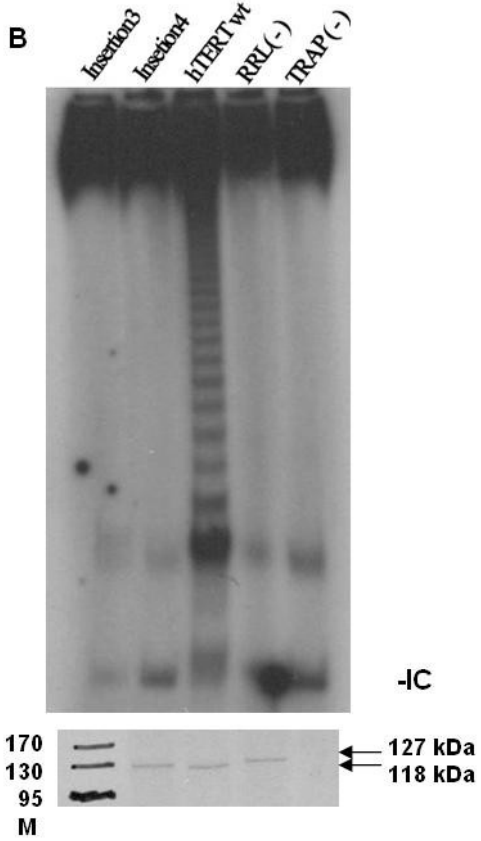
INS4 1051-NA GMCRCLASVAAVPACWC*



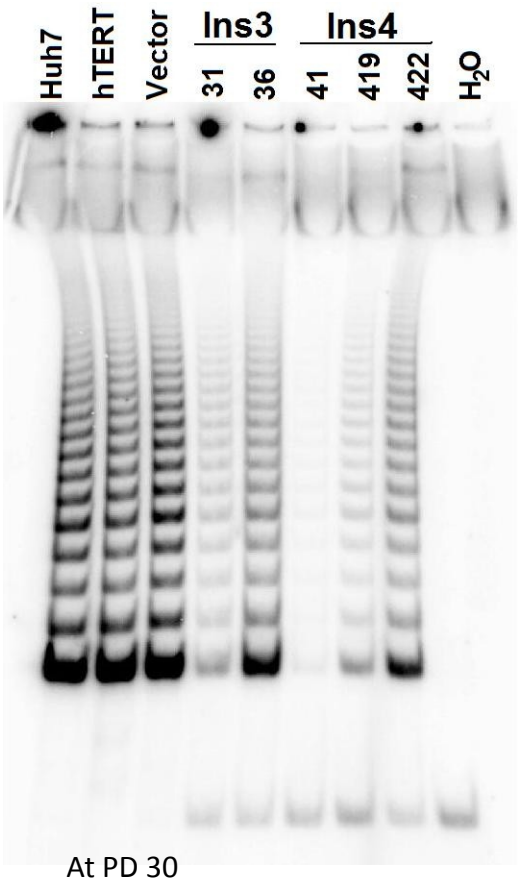
The *in vitro* INS3- and INS4-reconstituted enzymes do not express telomerase activity, likely due to the importance of the hTERT C-terminus for activity



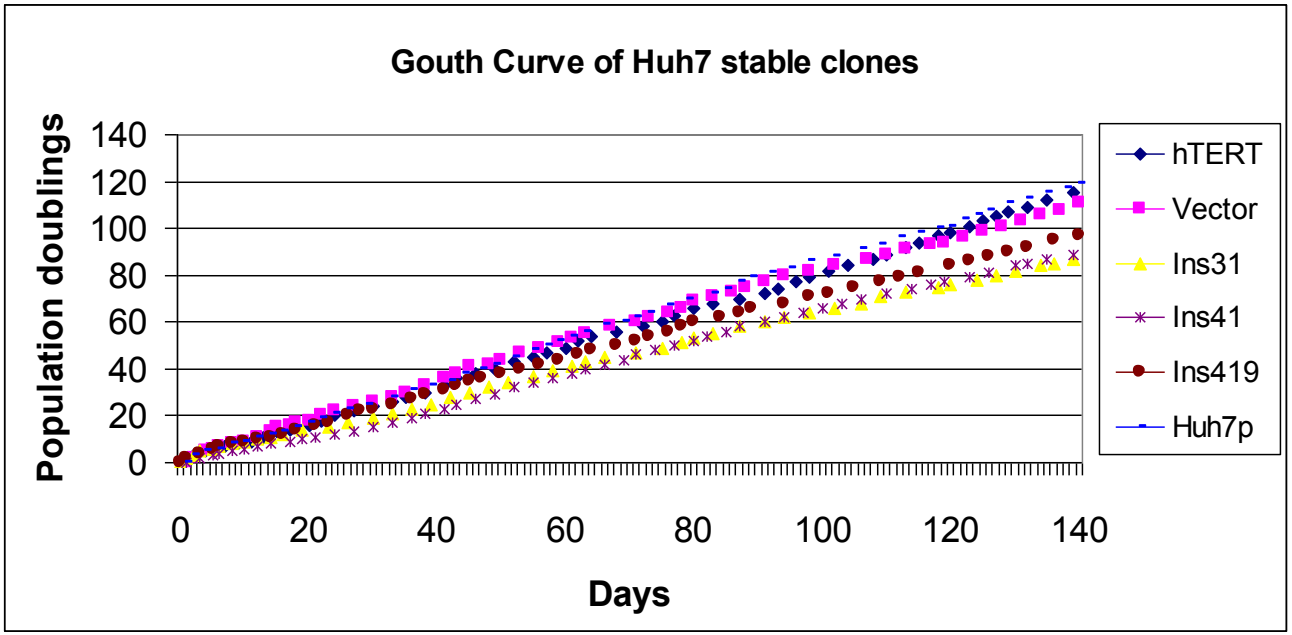
Confirmation of spliced mRNAs using polyA purified RNA from K562 leukemia cell line



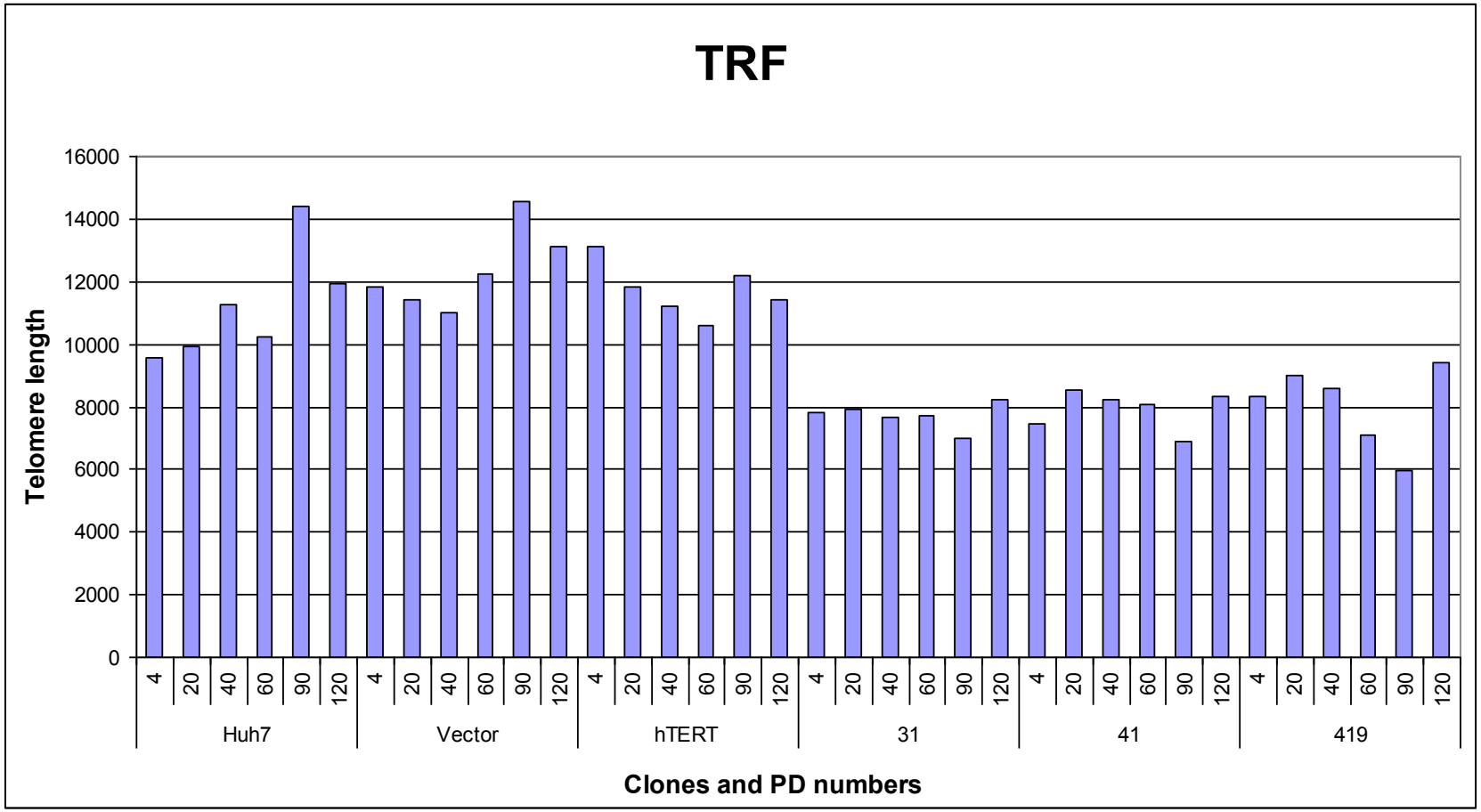
Stable expression of INS3 and INS4 variants in Huh7 hepatocarcinoma cell lines inhibits telomerase activity and slows cell growth



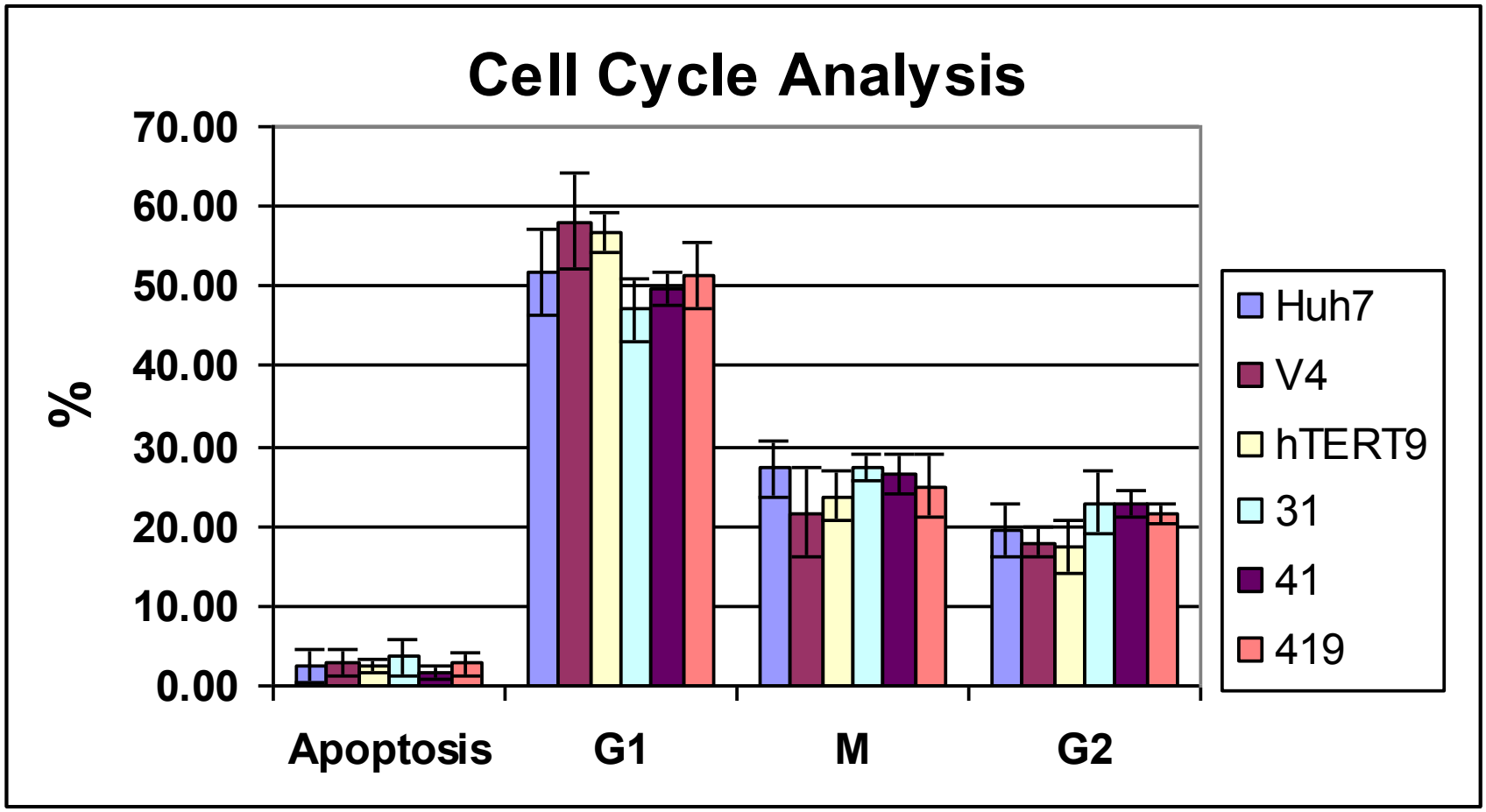
At PD 30



Ins 3 and Ins 4 stably expressing cell lines exhibit shorter telomeres compared to Huh7 parental cells and hTERT control, but telomeres do not progressively shorten with increasing population doubling

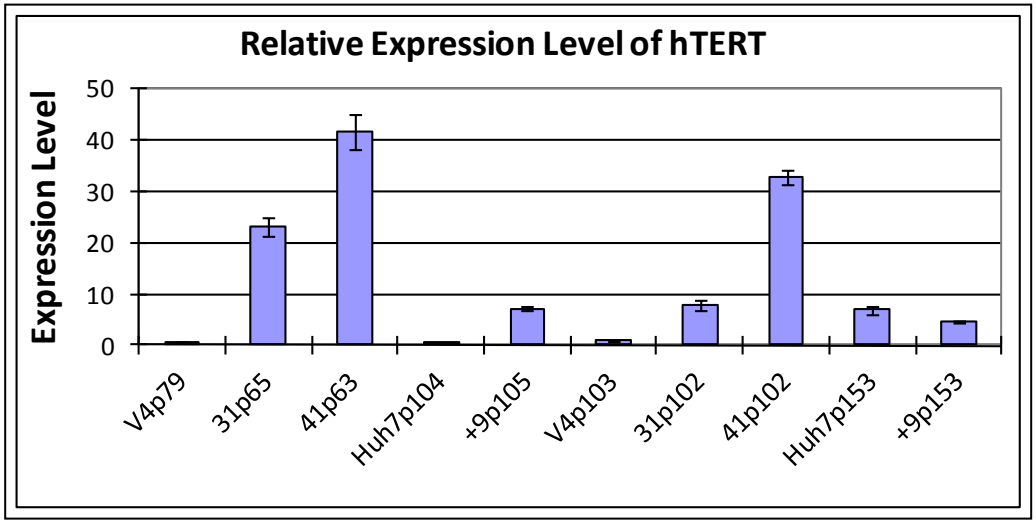


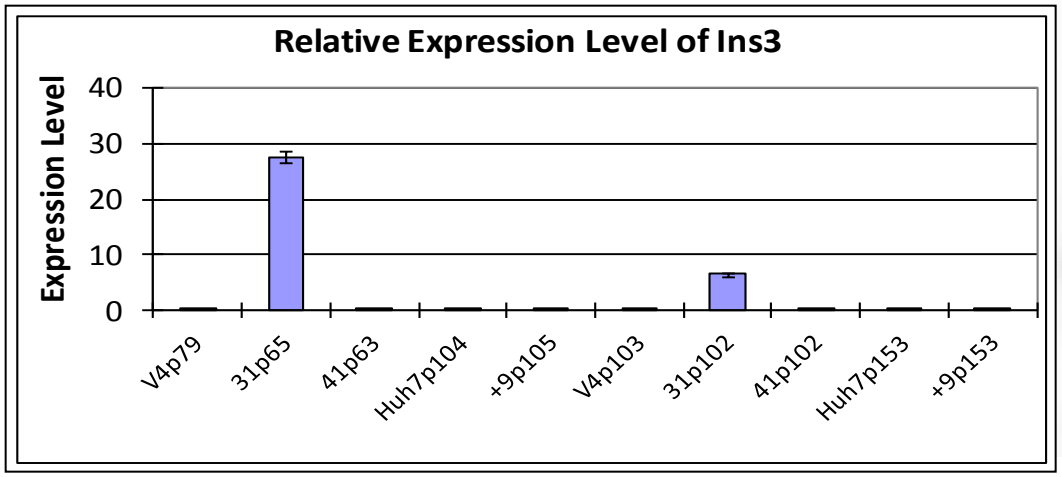
No significant difference in cell cycle profile or percentage apoptosis in cells stably expressing INS3 or INS4 variant compared to parental cells or cells expressing hTERT

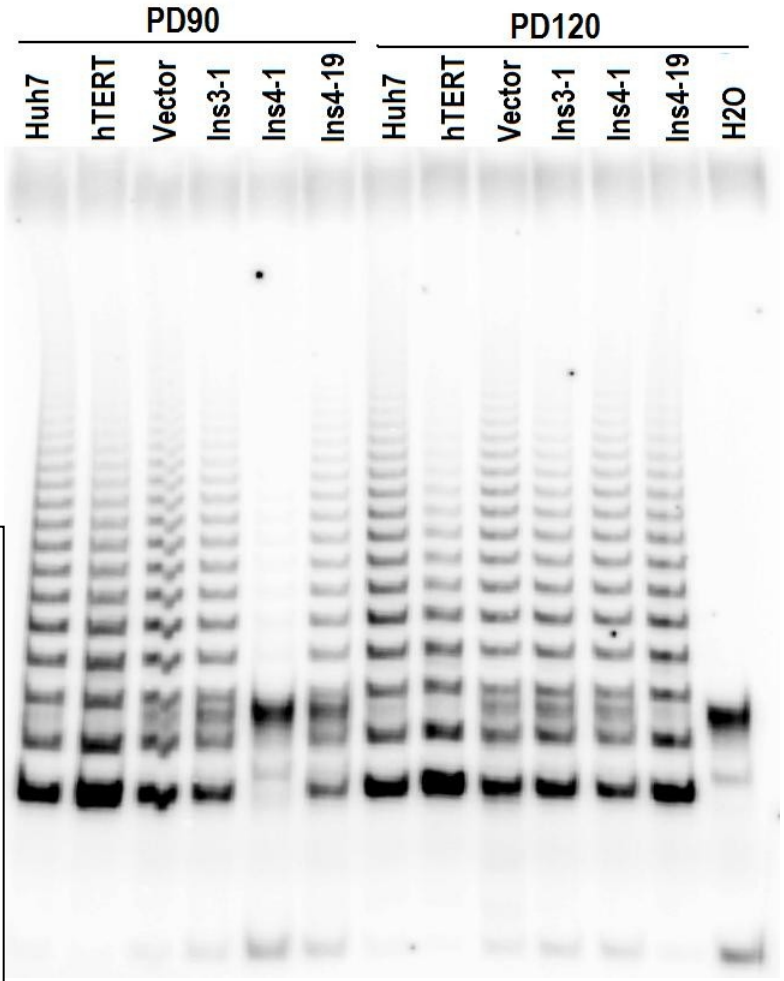


PD 60-80

Loss of Ins3 transgene expression results in loss of telomerase inhibition at late population doublings







Conclusion

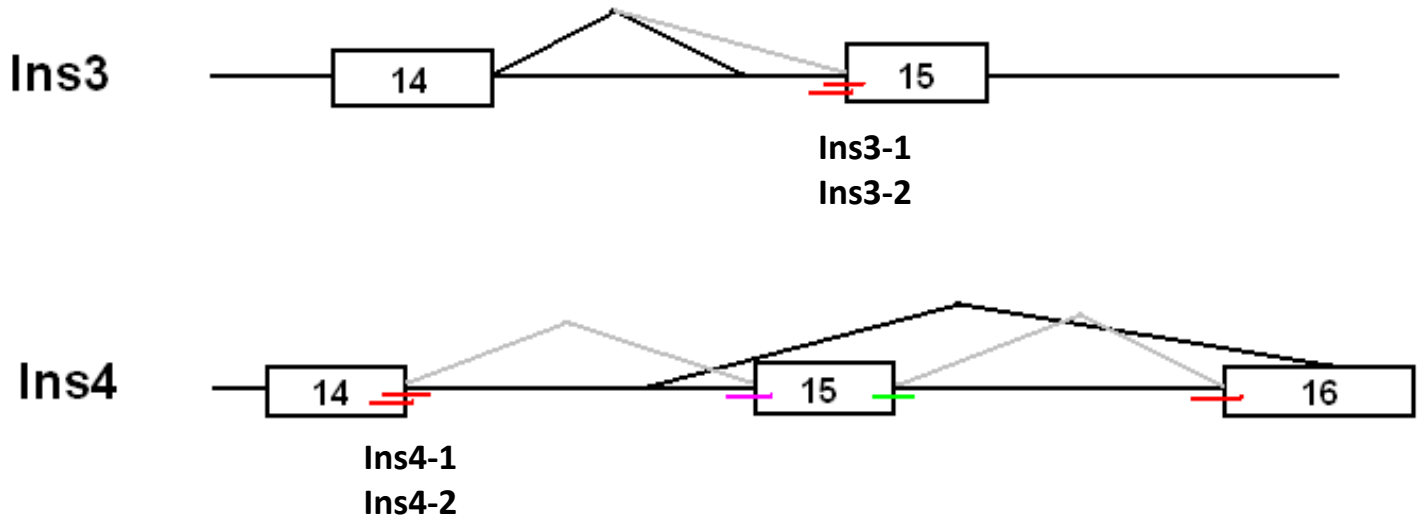
Ins3- and Ins4-reconstituted telomerases are inactive in vitro

Stable expression of Ins3 and Ins4 variants in telomerase positive Huh7 leads to telomerase inhibition, slowed growth and shorter telomere lengths, but no significant changes in cell cycle profile

Telomerase inhibition is not maintained at late population doublings, likely due to loss of expression of the Ins3 transgene expression

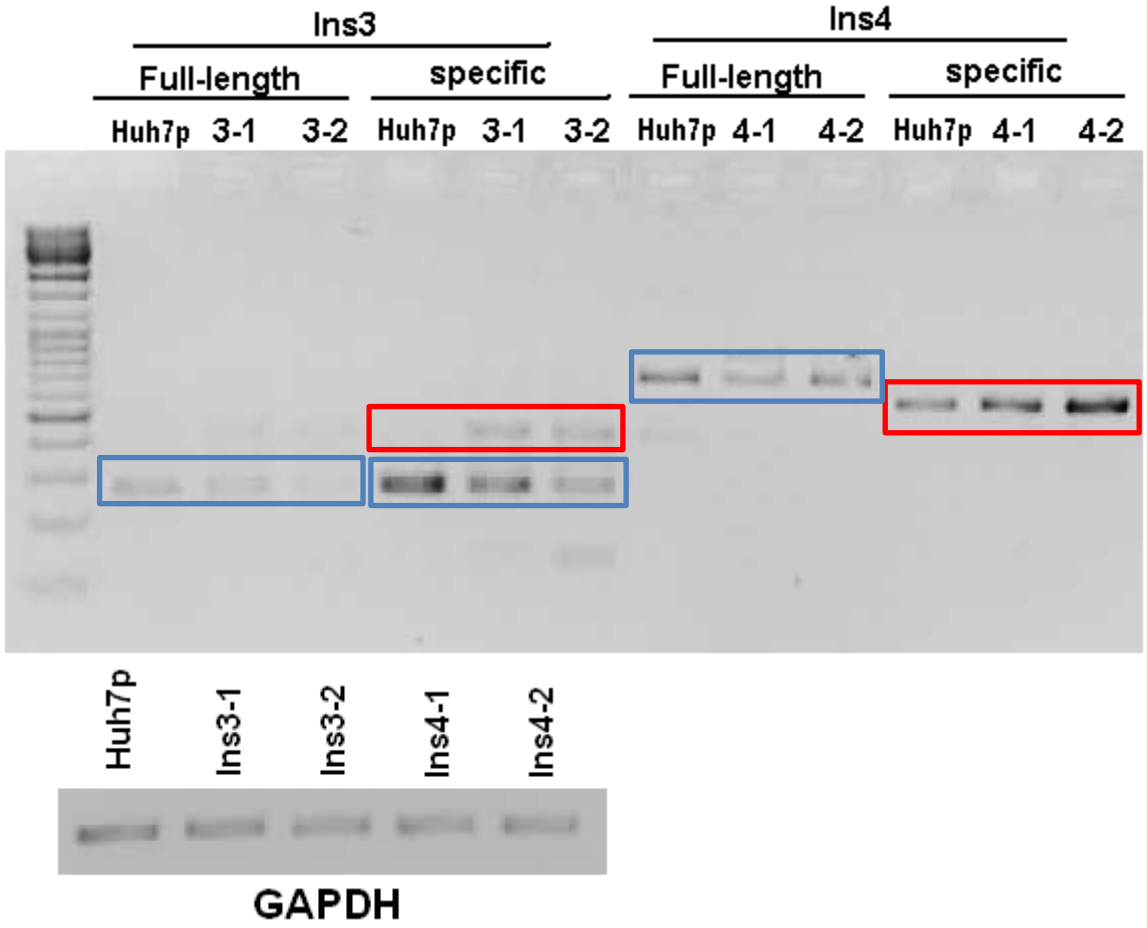
Antisense oligonucleotide-mediated modulation of Ins3 and Ins4 splicing

-Investigated in the context of genetic diseases to prevent aberrant splicing of genes
-With cancer-related genes has been exploited to redirect the splicing of an anti-apoptotic to a pro-apoptotic Bcl-x variant
-One report of telomerase and cell growth inhibition upon oligomer-mediated modulation of hTERT alternative splicing in prostate cancer cells (Brambilla, C. et al. 2004. CMLS 61, 1764-1774)

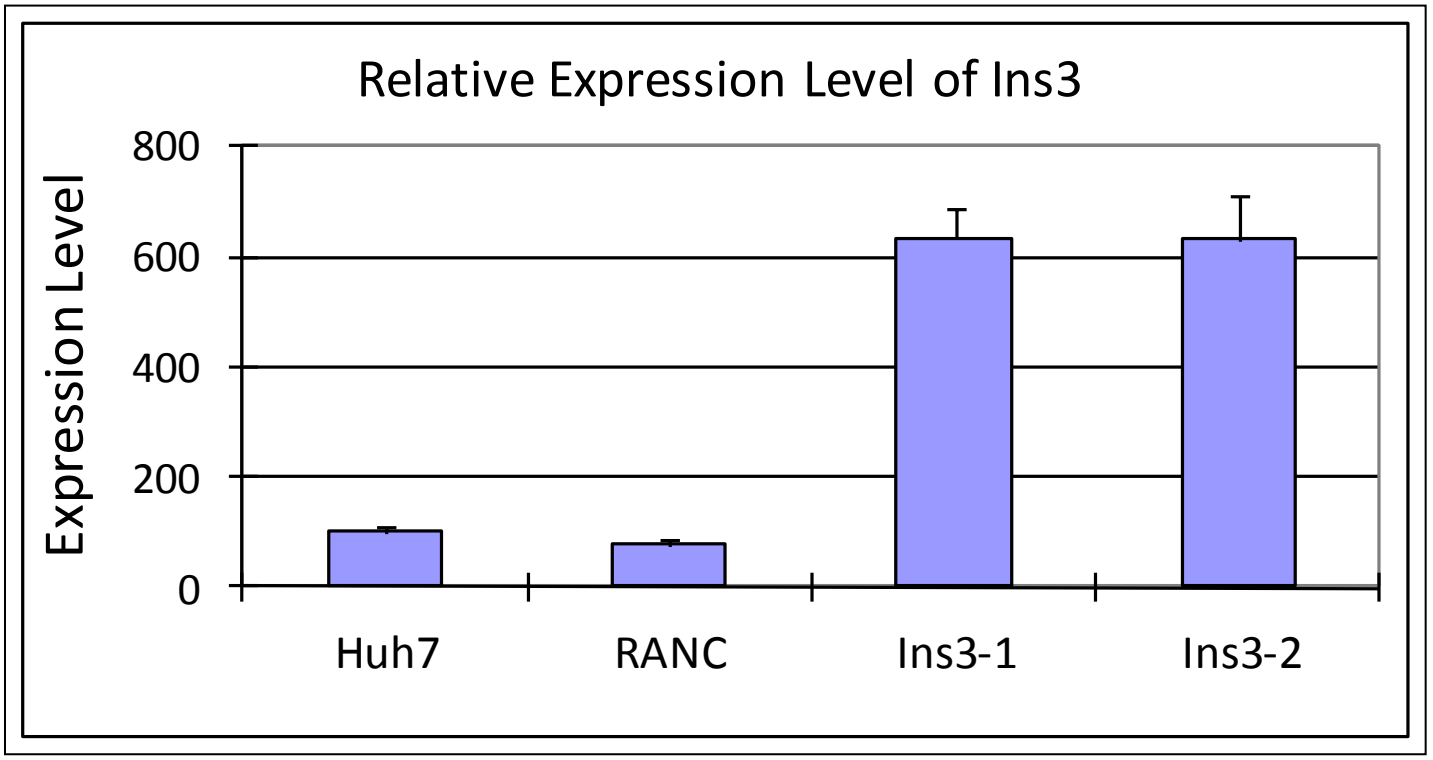


Used 2'-O-methyl-RNA phosphorothioate oligonucleotides

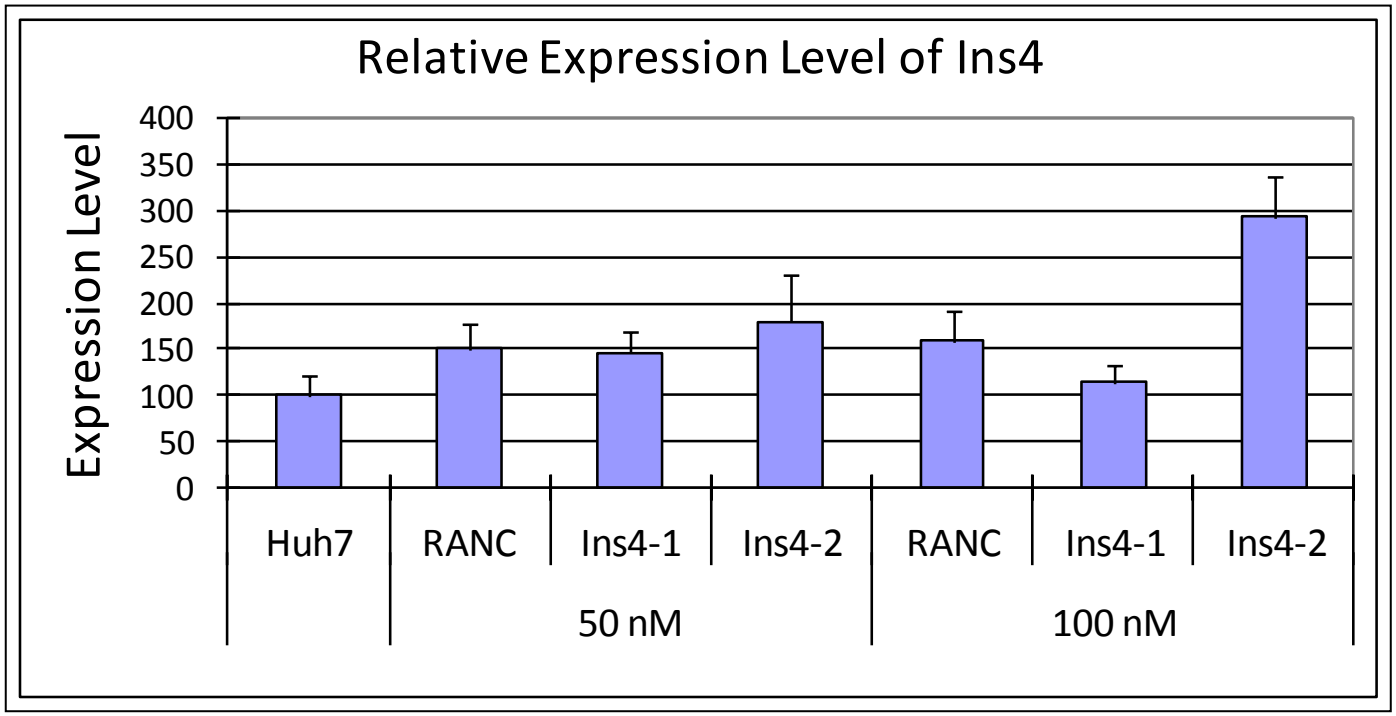
Increased expression of Ins3 and Ins4 spliced variants and decreased expression of full length hTERT mRNA upon antisense oligonucleotide treatment



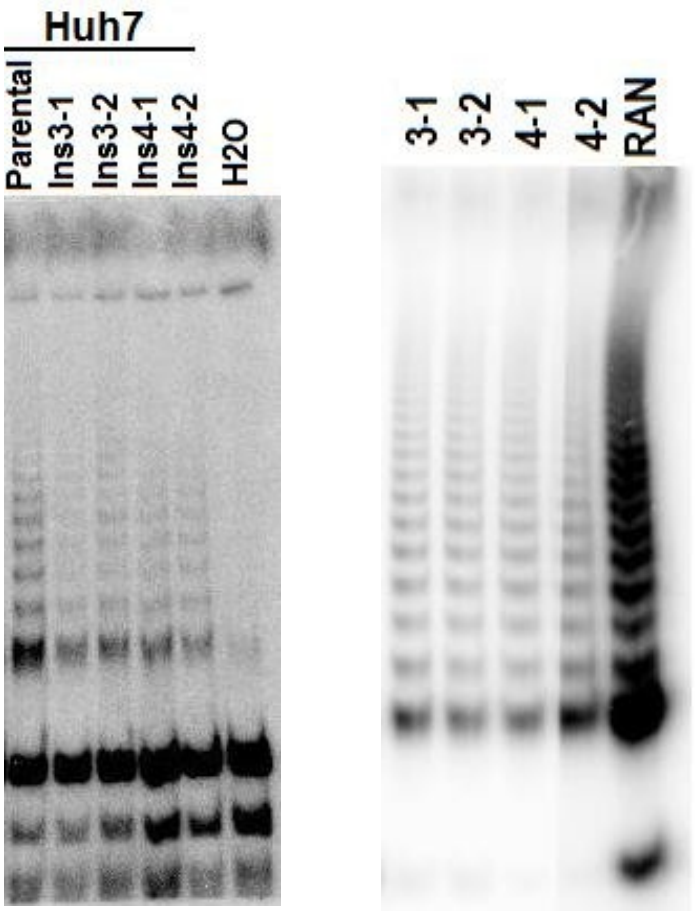
Quantitation of endogenous Ins3 levels by Real-time PCR



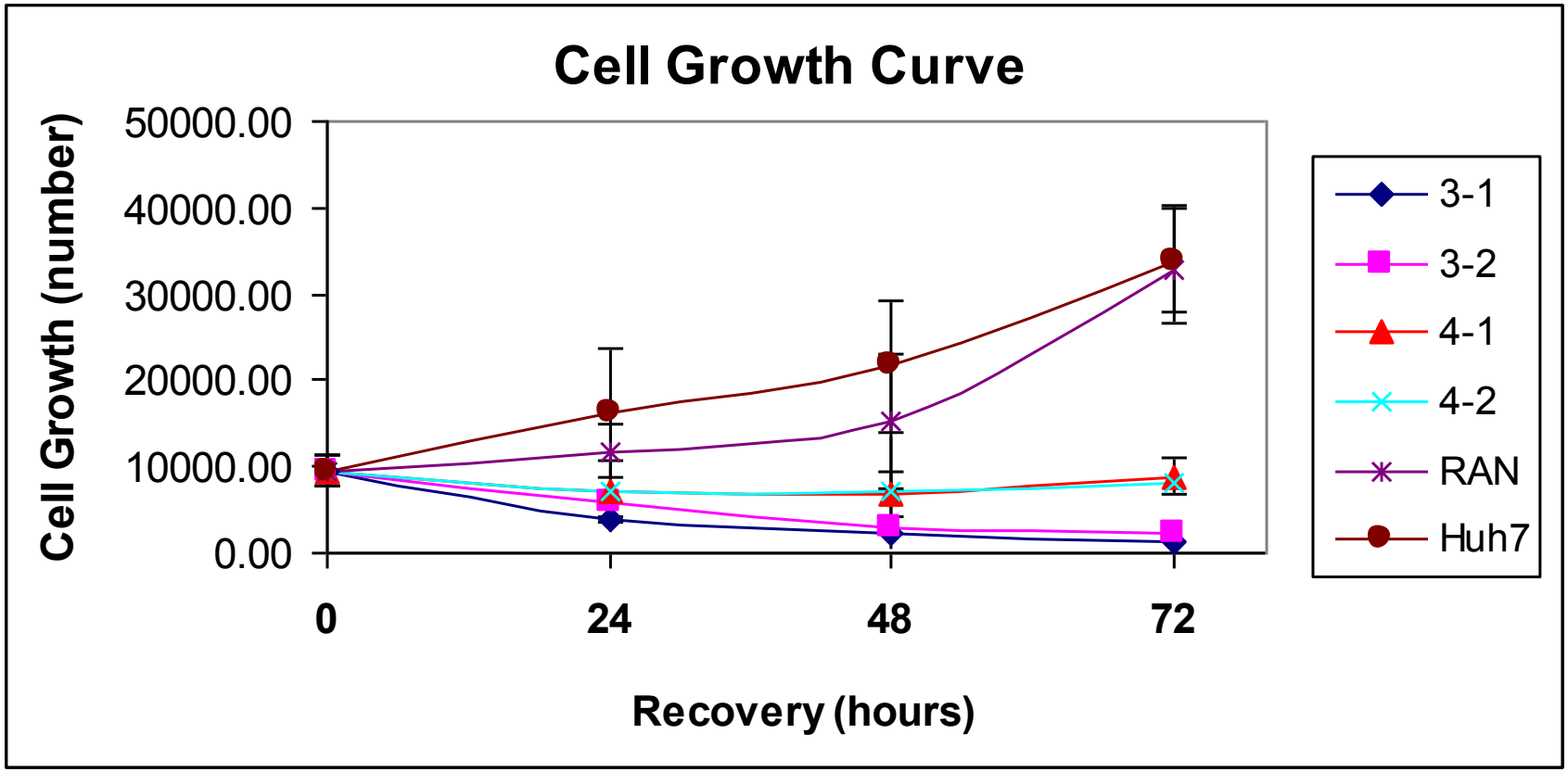
Quantitation of endogenous Ins4 levels by Real-time PCR



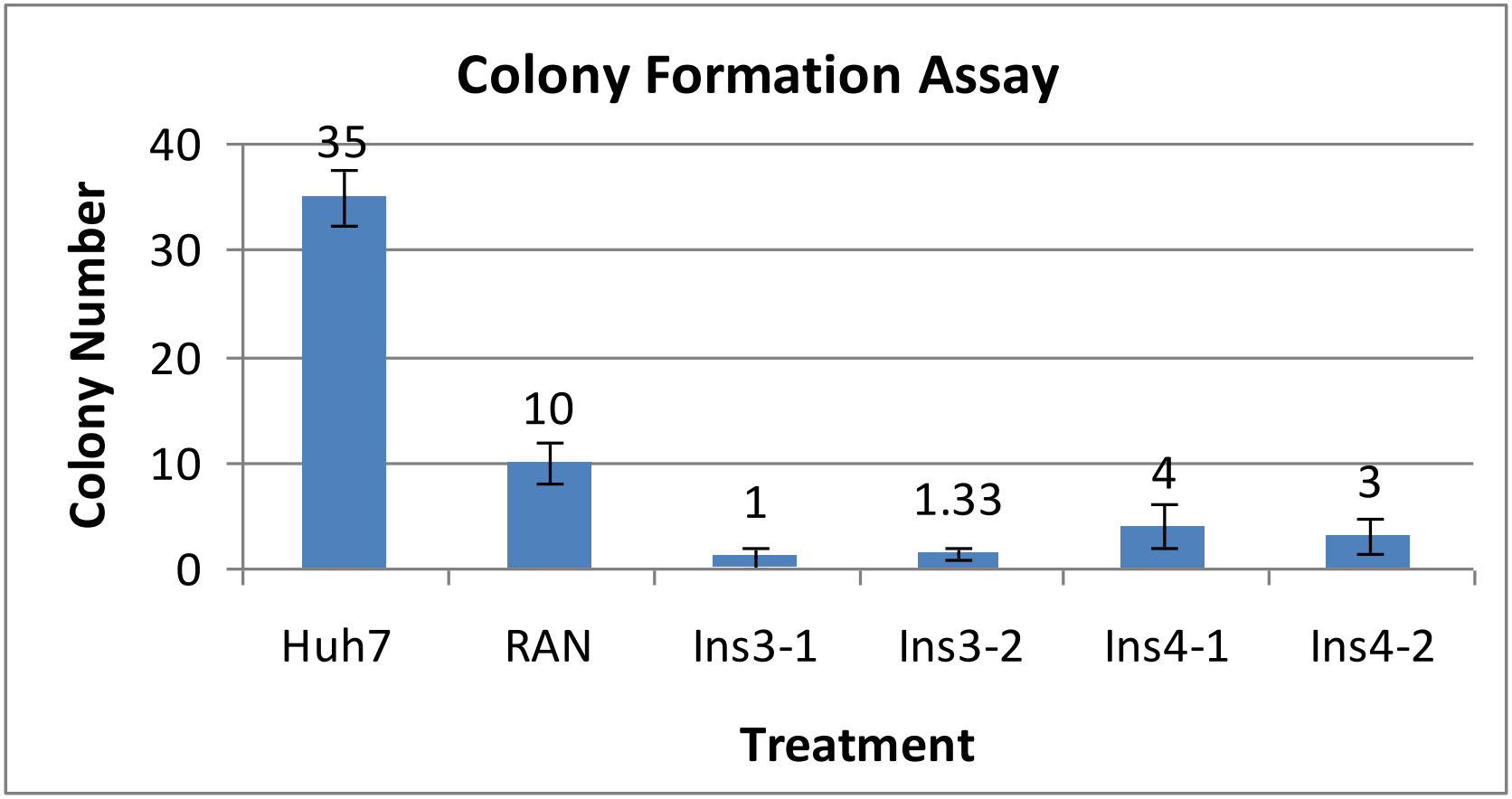
Increased expression of Ins3 and Ins4 spliced variants and decreased expression of full length hTERT mRNA reduces telomerase activity



Growth inhibition upon antisense oligonucleotide treatment



Antisense oligonucleotide treatment decreased cell proliferation as measured by colony formation ability



Conclusion

Antisense oligomer-mediated expression of Ins3 and Ins4 spliced hTERT mRNAs:

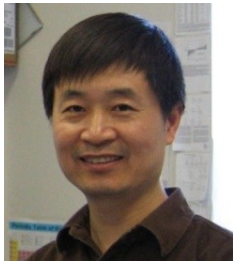
Inhibited endogenous hTERT expression in Huh7 cells
Inhibited endogenous telomerase activity in Huh7 cells
Inhibited the growth, viability and proliferation of Huh7 cells

Perspective

Short term effects are most likely telomere-shortening independent and may be due to the loss of telomere protection

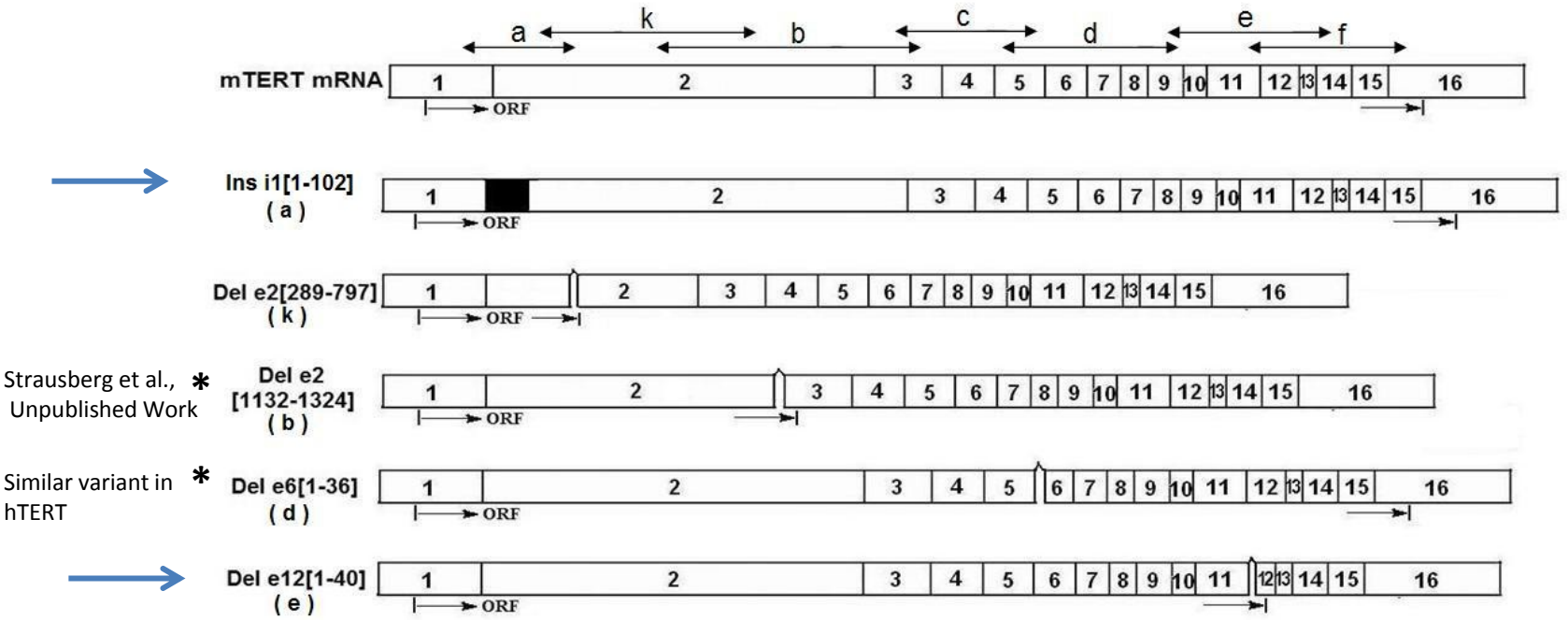
Investigate if apoptosis is induced

Verify off target effects using hTERT-negative cell line



Identification and characterization of alternatively-spliced mTERT mRNAs

mTERT Alternatively-Spliced Variants



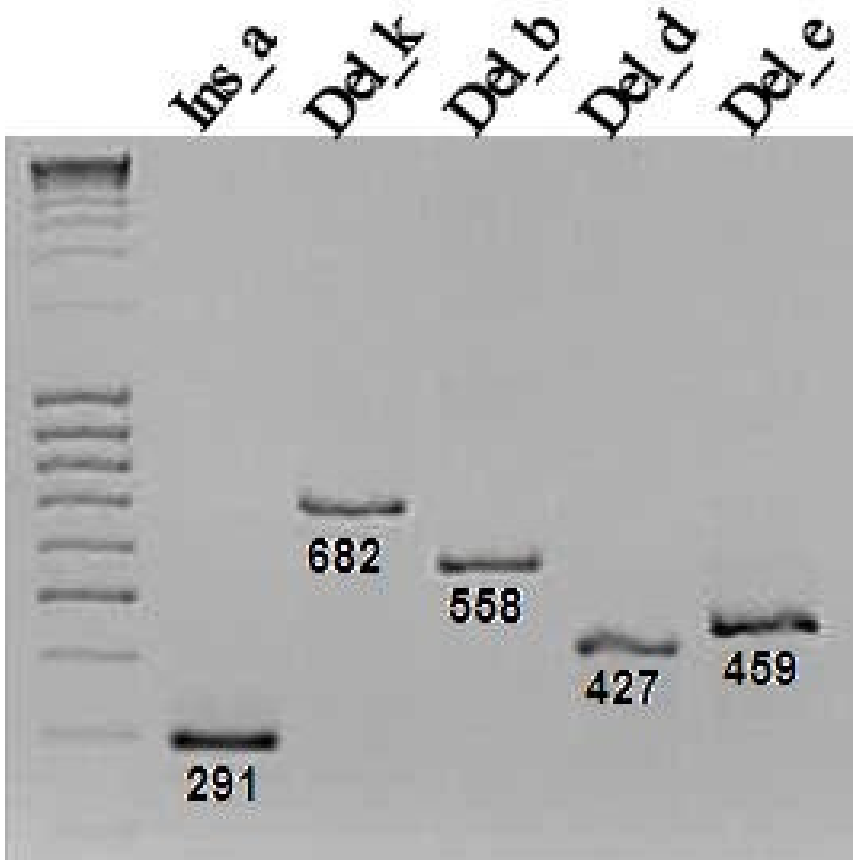
Five of the suspected variants were confirmed by sequencing.

- Exons = white boxes with numbering inside.
- Arrowheads above full-length *mTERT* mRNA = the regions of each PCR product covered.
- mTERT ASPSs have been assigned descriptive names.

CB17: mouse fibroblast cell line
NIH3T3: Mouse embryonic fibroblast
FM3A: mouse mammary carcinoma

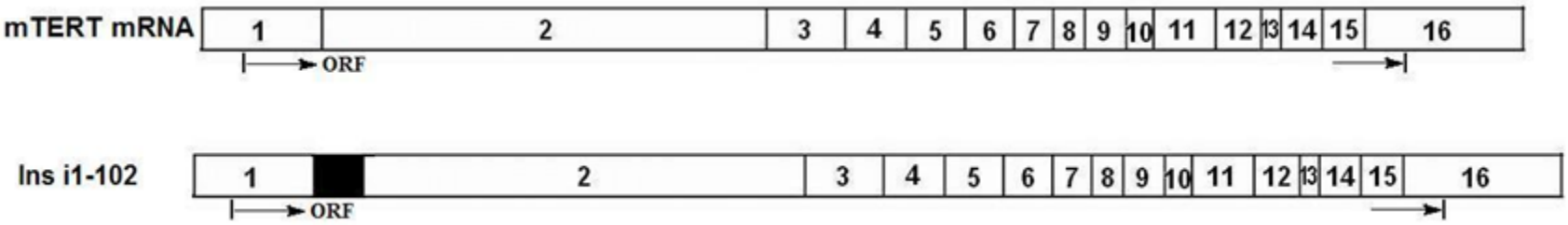
mTERT Alternatively-Spliced Variants

Variant	Primers used for PCR 1	Primers used for PCR 2	Expected Size of Product (base pairs)
Ins a	InsaF 1069R	InsaF 500R	291
Del k	DelkF 2092R	DelkF 1689R	682
Del b	806F DelR2	DelbF 2092R	558
Del d	DeldF 3056R	DeldF DelR2	427
Del e	DeleF Ins4-R3	DeleF Ins3-R2	459

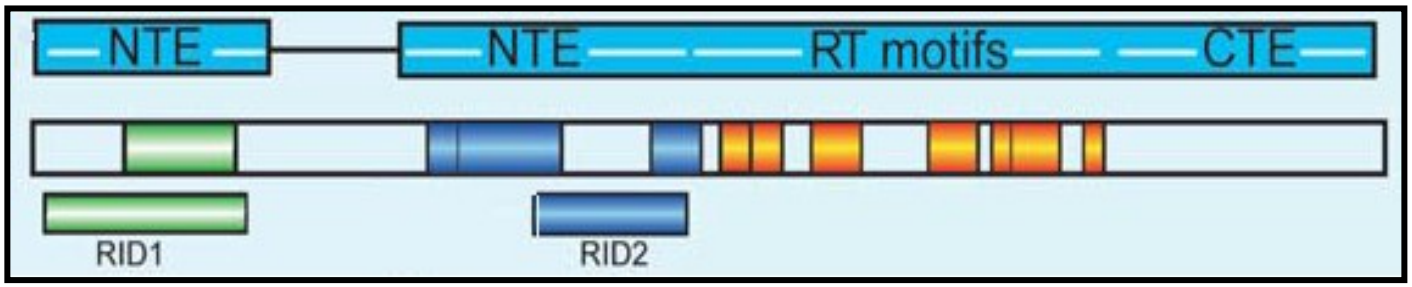


Generated PCR products of alternatively spliced mRNA using **splicing variant-specific** primers and polyA purified RNA from NIH3T3 cells

Insertion Variant, Insi1

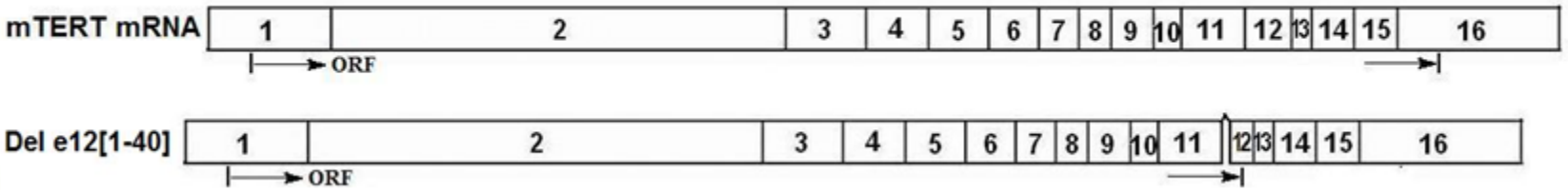


- Found in intron 1
- Ins i1 is in a region which corresponds to the hTERT RID1 motif.

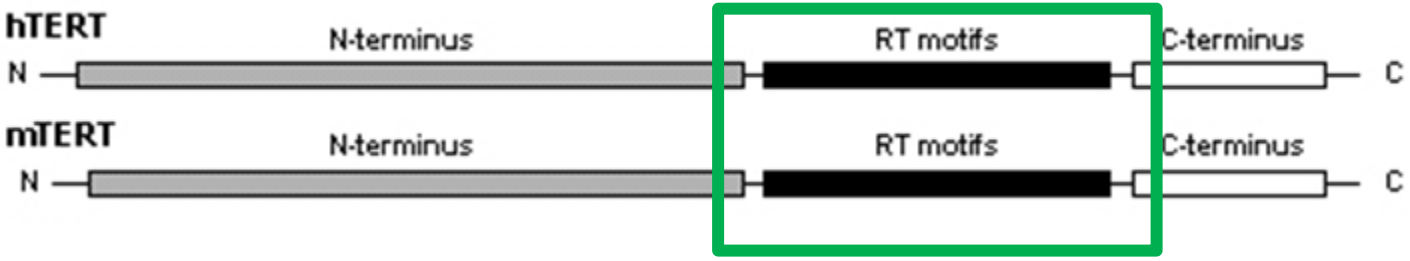


The telomerase reverse transcriptase (TERT) is divided into three regions, the TERT essential N-terminal (NTE) domain, the reverse-transcriptase (RT) motifs and the TERT C-terminal extension (CTE). Adapted from (Autexier and Lue, 2006.)

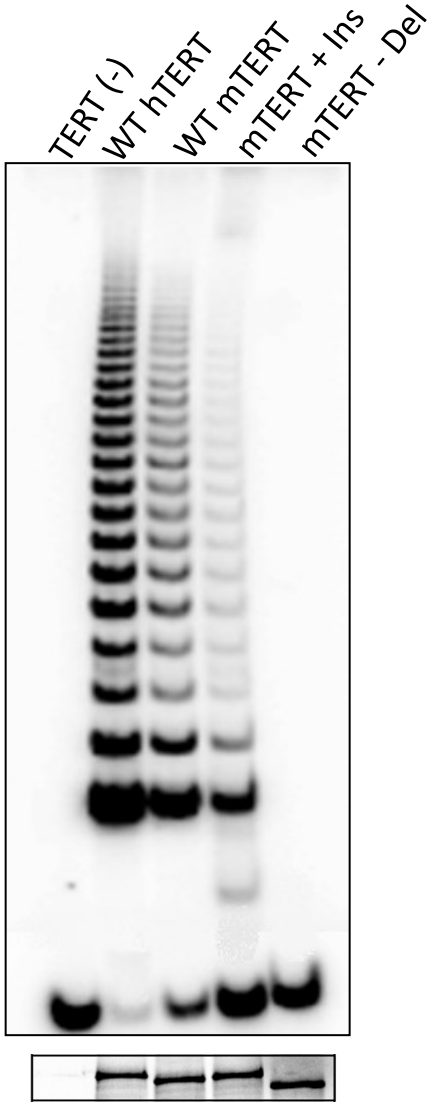
Deletion Variant, Dele12

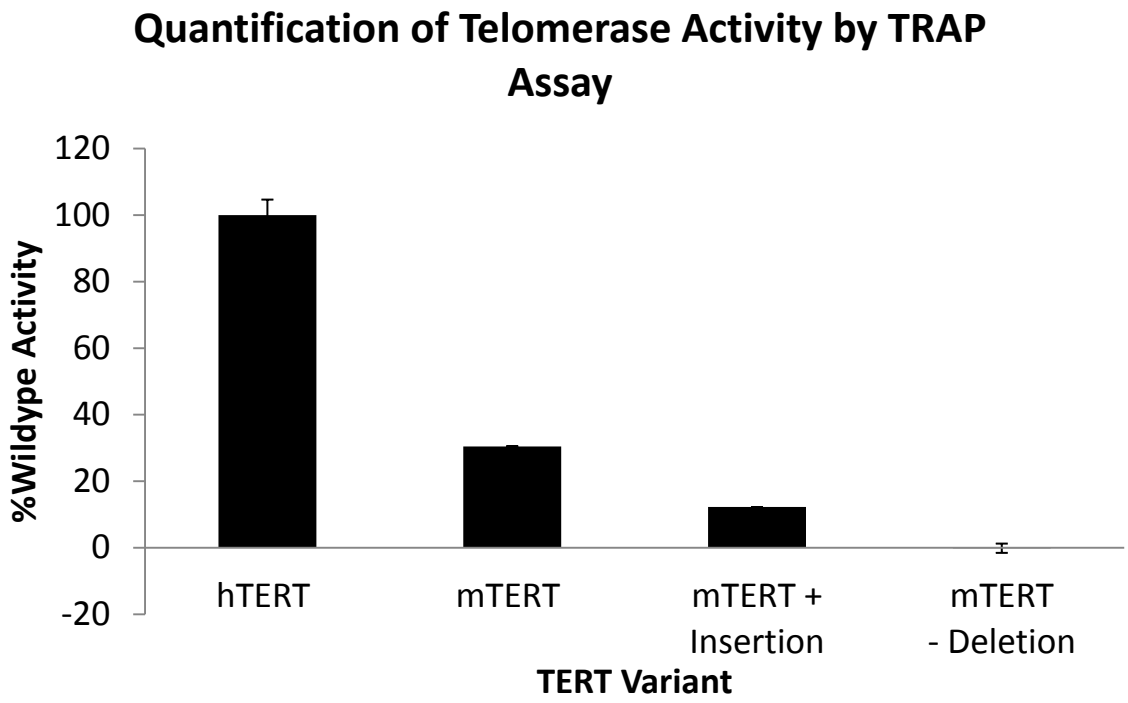


- Deletion falls in a region which corresponds to the hTERT CTE
- Deleting a sequence in such an essential part of the mTERT may modify it slightly or it might render it completely inactive.

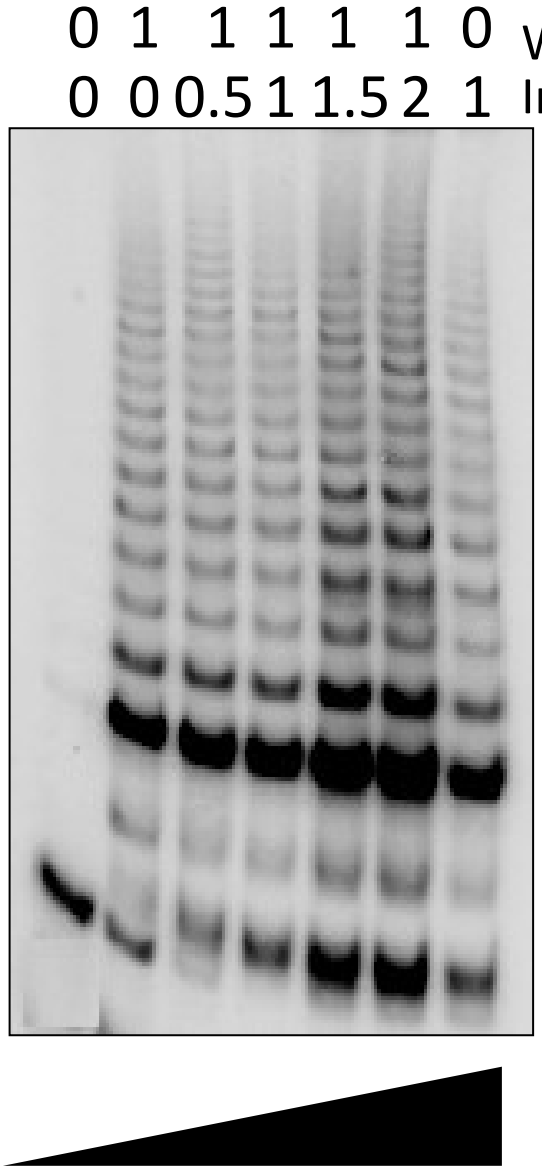


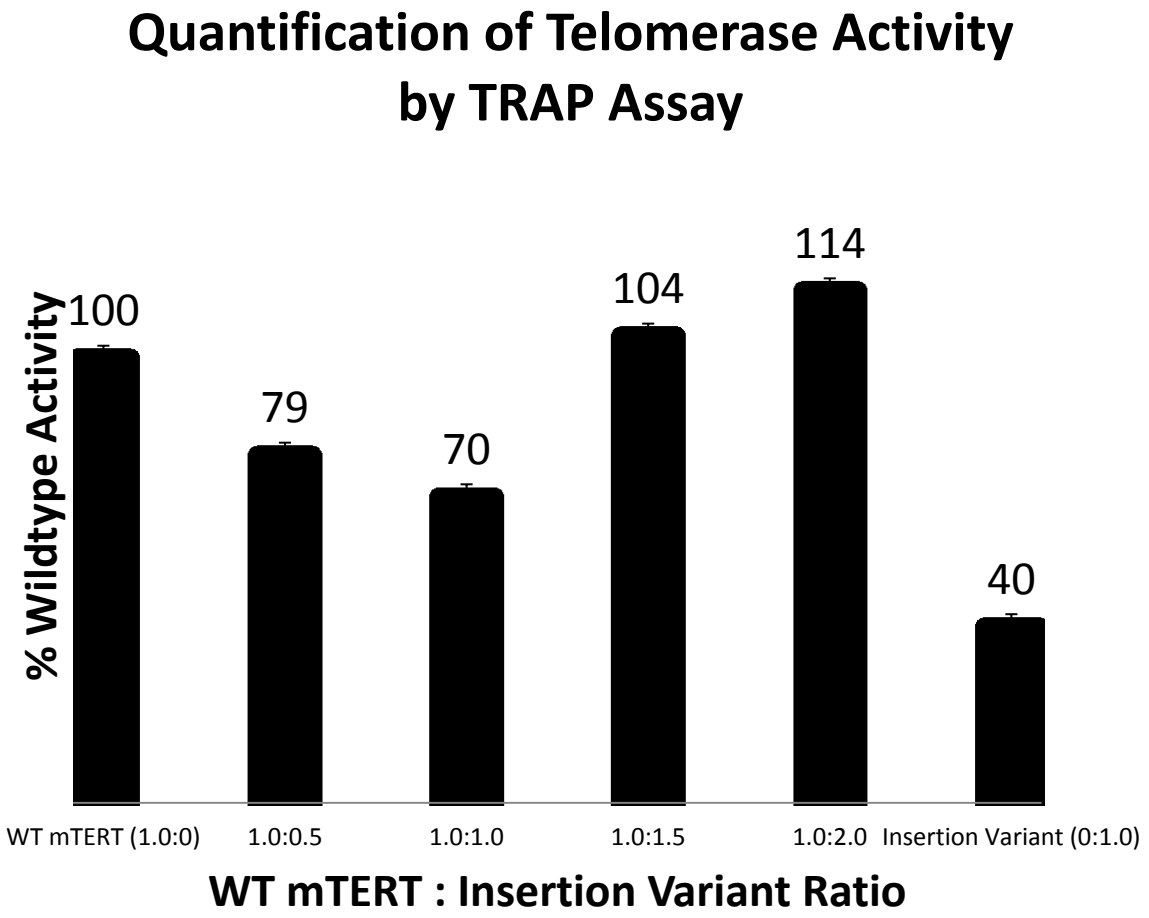
mTERT insertion but not the deletion variant reconstitutes telomerase activity



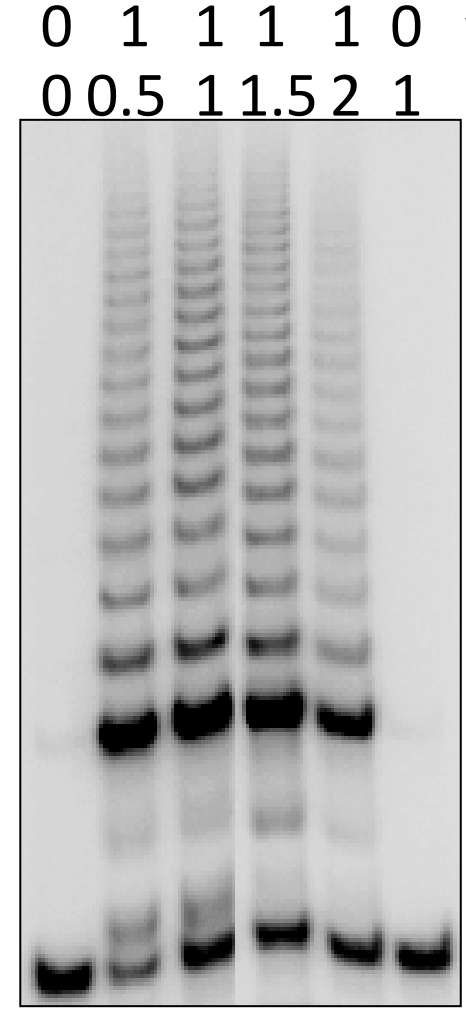


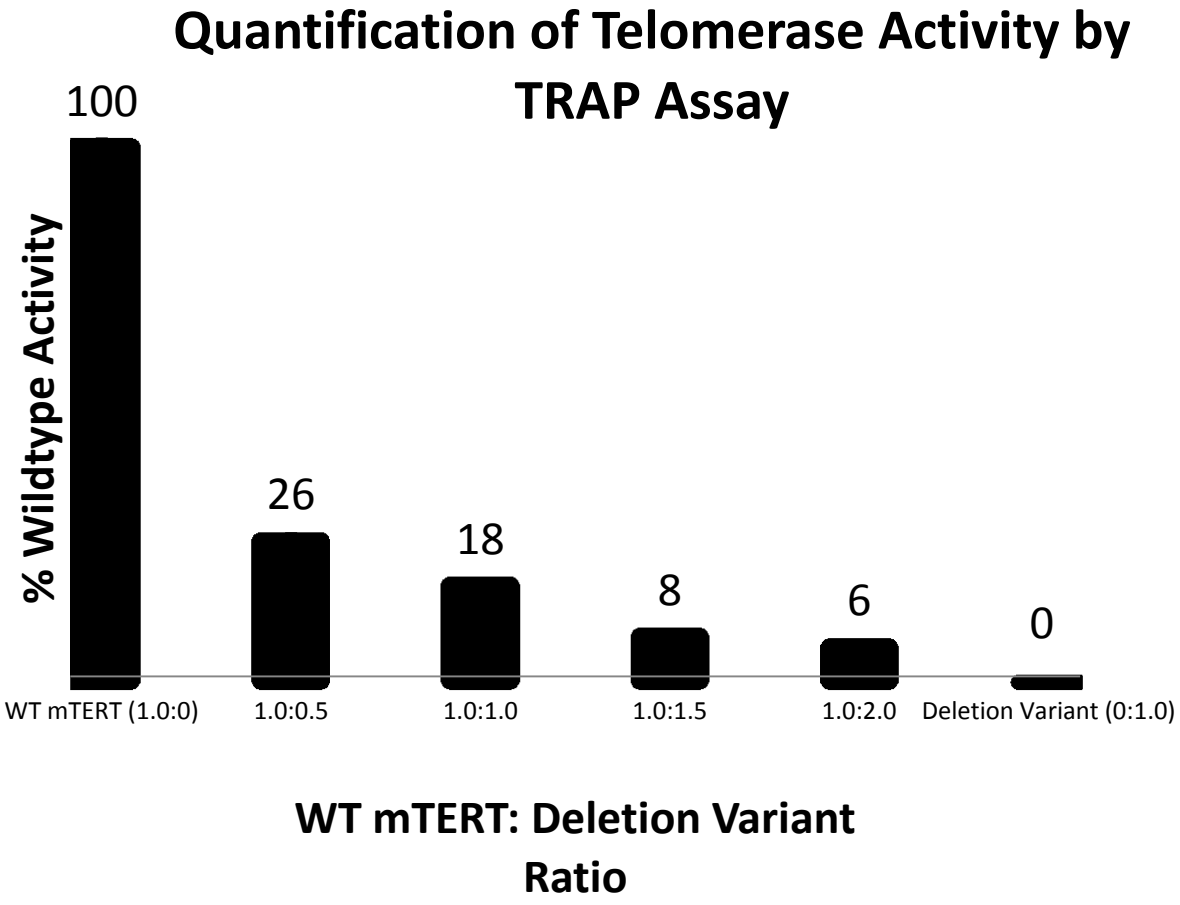
Telomerase activity of mixed wildtype mTERT/insertion variant suggests the insertion variant has no inhibitory effects on wildtype mTERT



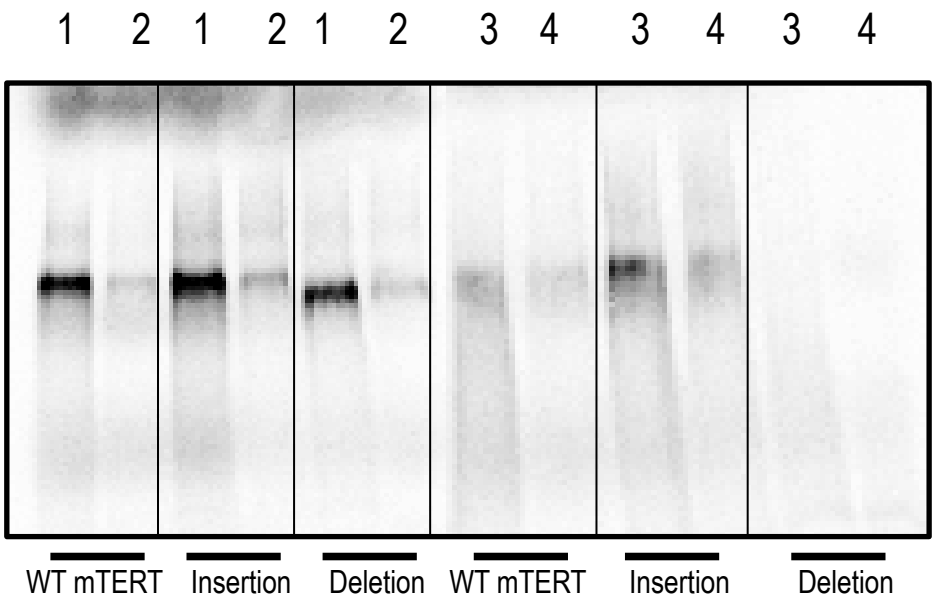


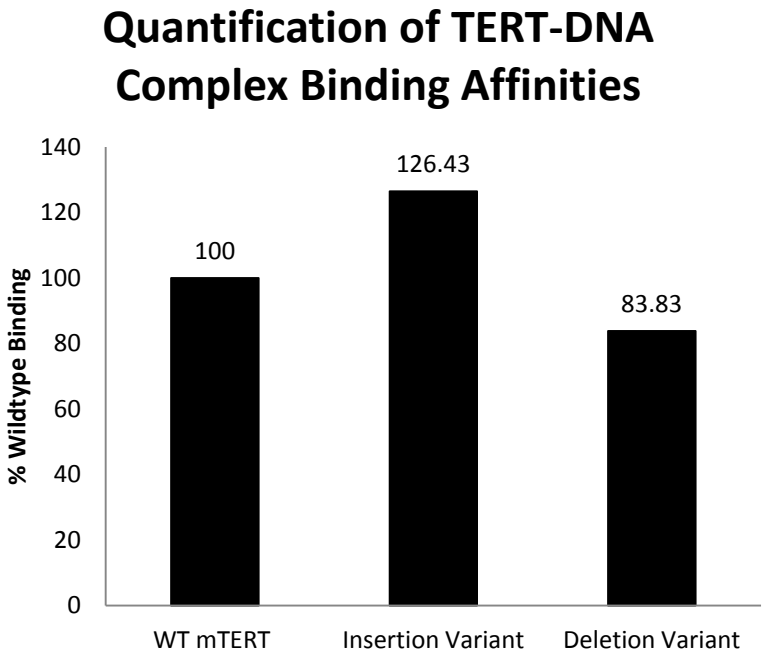
Telomerase activity of mixed wildtype mTERT/deletion variant the deletion variant seems to be inhibiting telomerase activity and may have dominant negative effects on telomerase activity





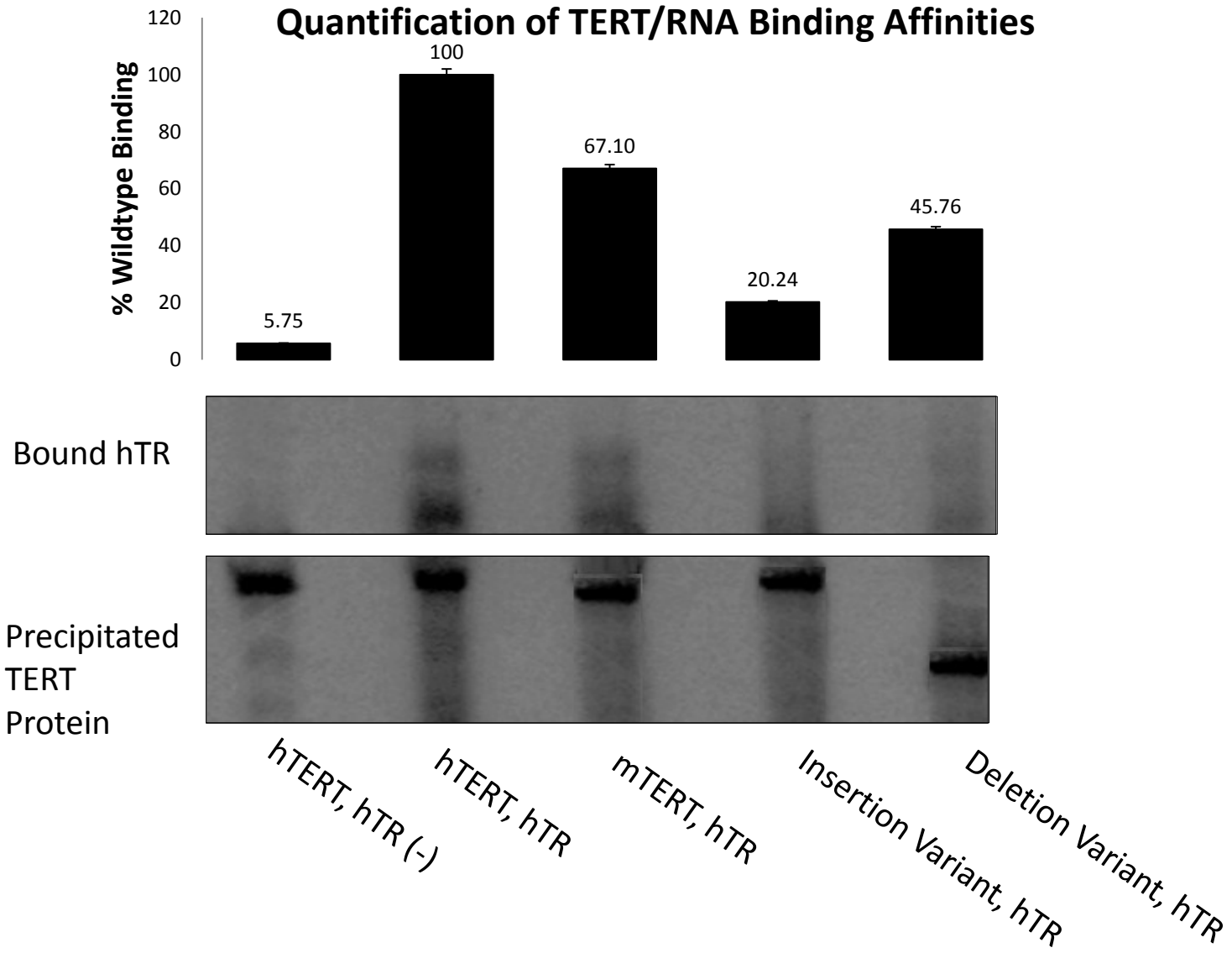
Insertion and deletion variants do not appear to have DNA-binding defects in vitro





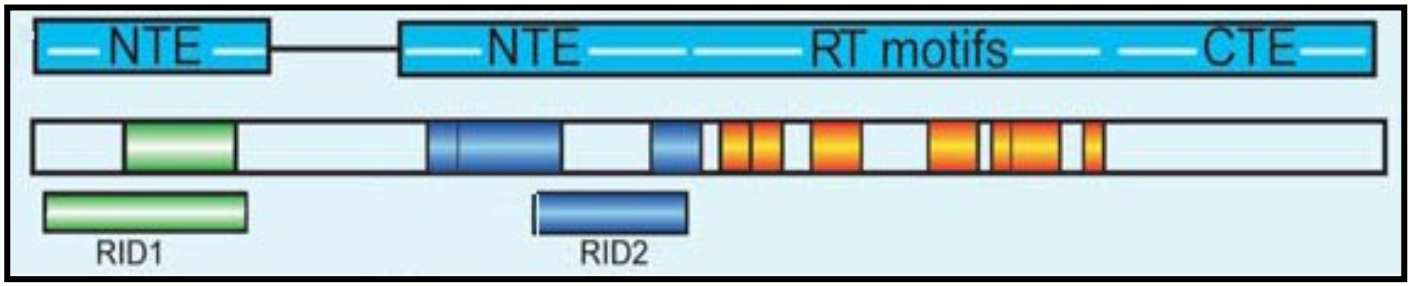
1) 5'-biotinylated primer
2) 5'-biotinylated antisense primer
3) Non-biotinylated primer
4) No primer

Both variants exhibit RNA-binding defects in vitro



RID1 Motif and RNA Binding

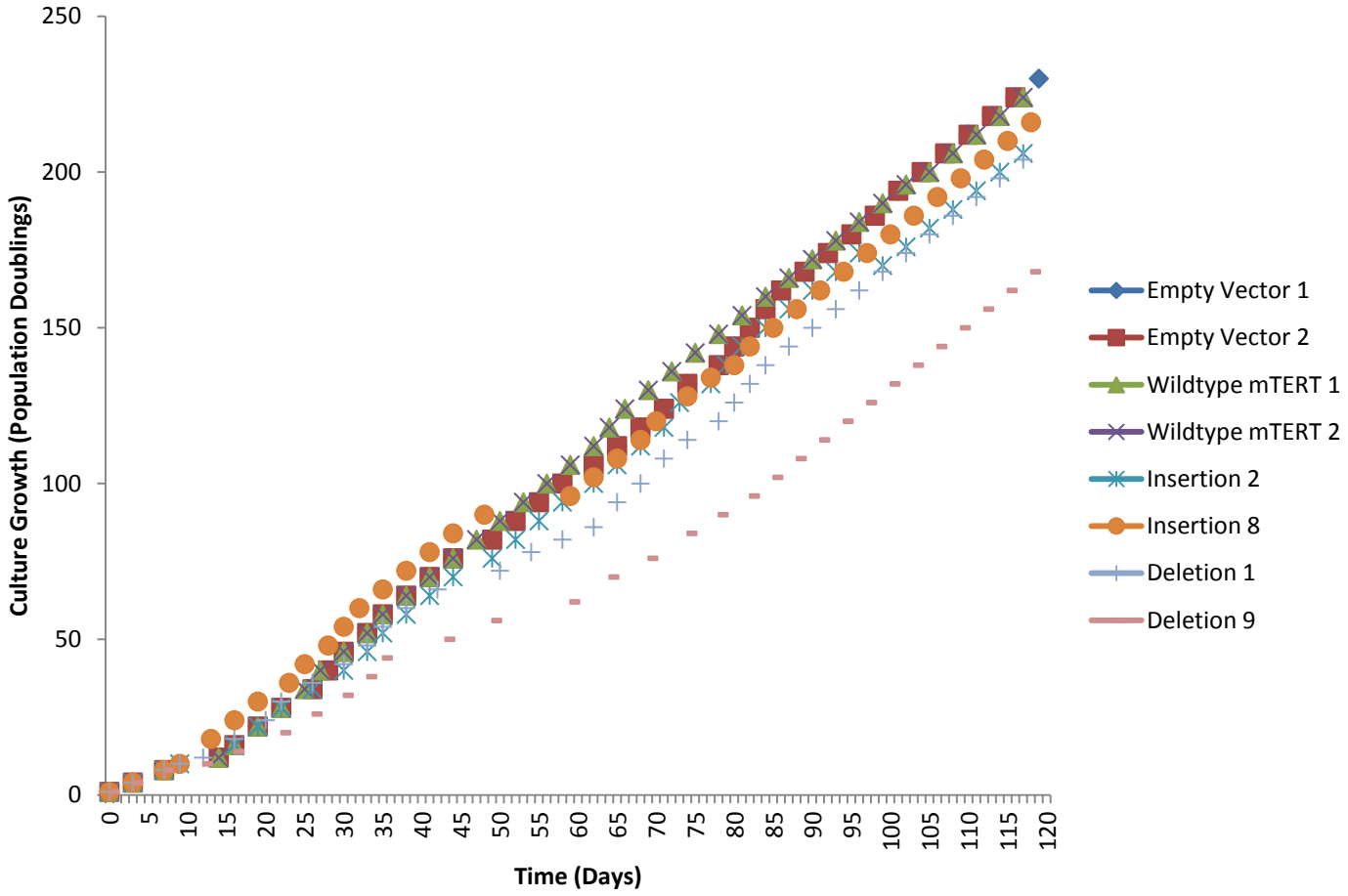
- A major accessory domain containing the **RNA-interaction domain 1 (RID1)**
- RID1 interacts with the hTR pseudoknot-template domain and hTERT's RT motifs and putative thumb and was shown to be essential for processivity, but not DNA synthesis.



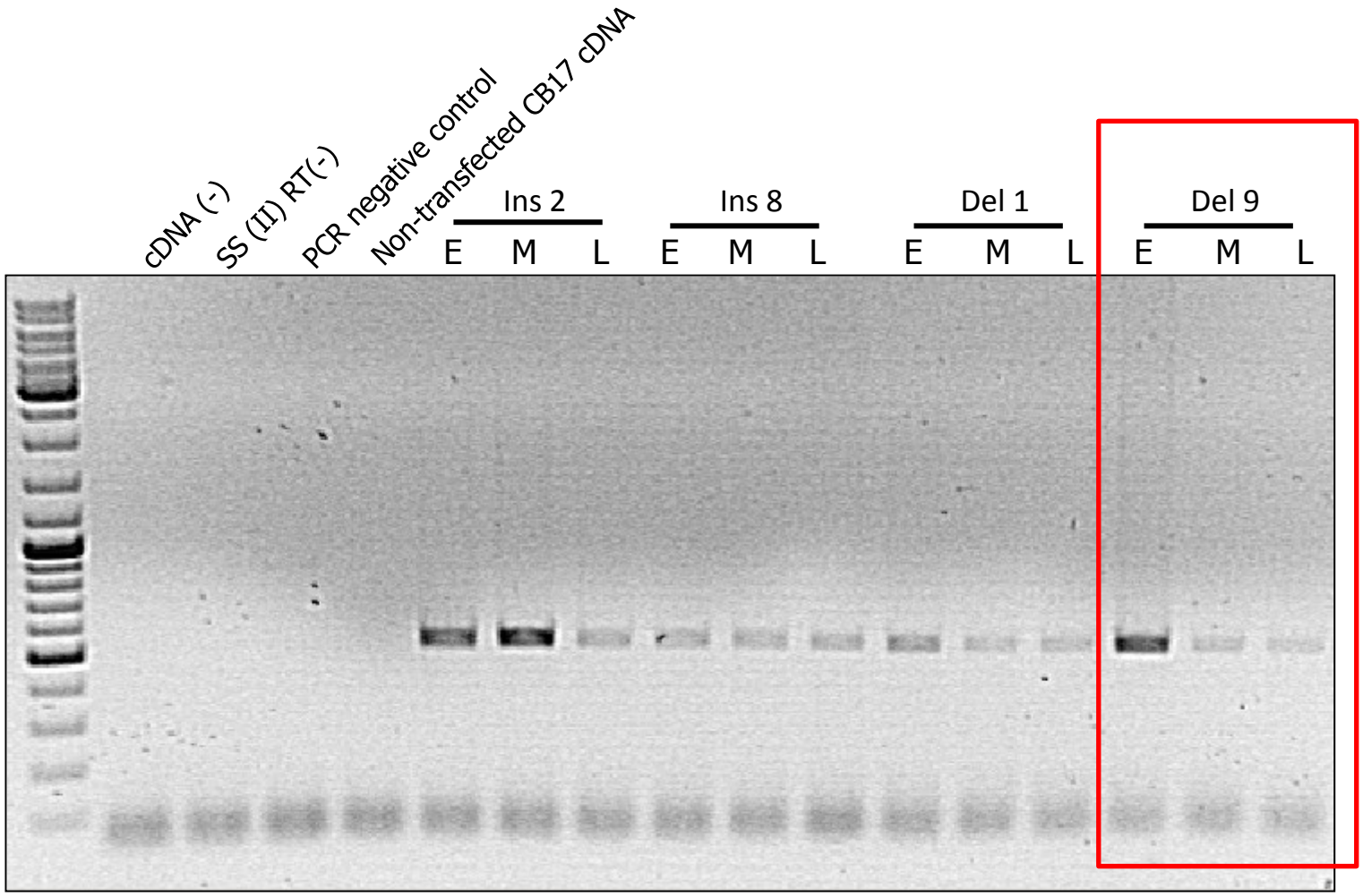
Autexier and Lue, 2006

- These preliminary results suggest that both the insertion and deletion variants have decreased binding affinities.
- For the former, the decrease in binding may be due to the fact that the 102-nucleotide insert falls in a region which corresponds to the human RID1 motif.

Stable expression of the deletion variant in the CB17 mouse fibroblast cell line slows cell growth

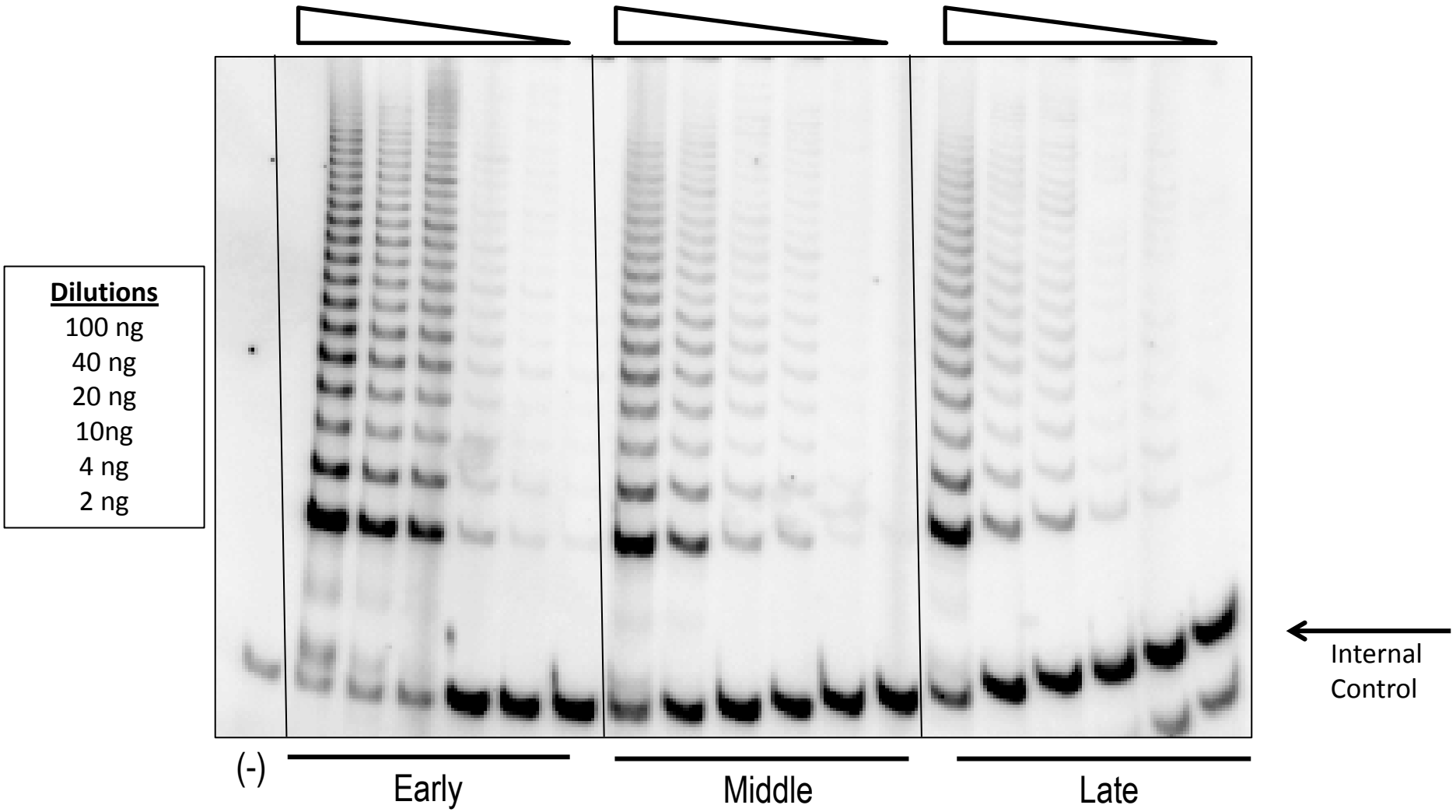


Alternatively spliced variants are stably expressed with increasing population doublings

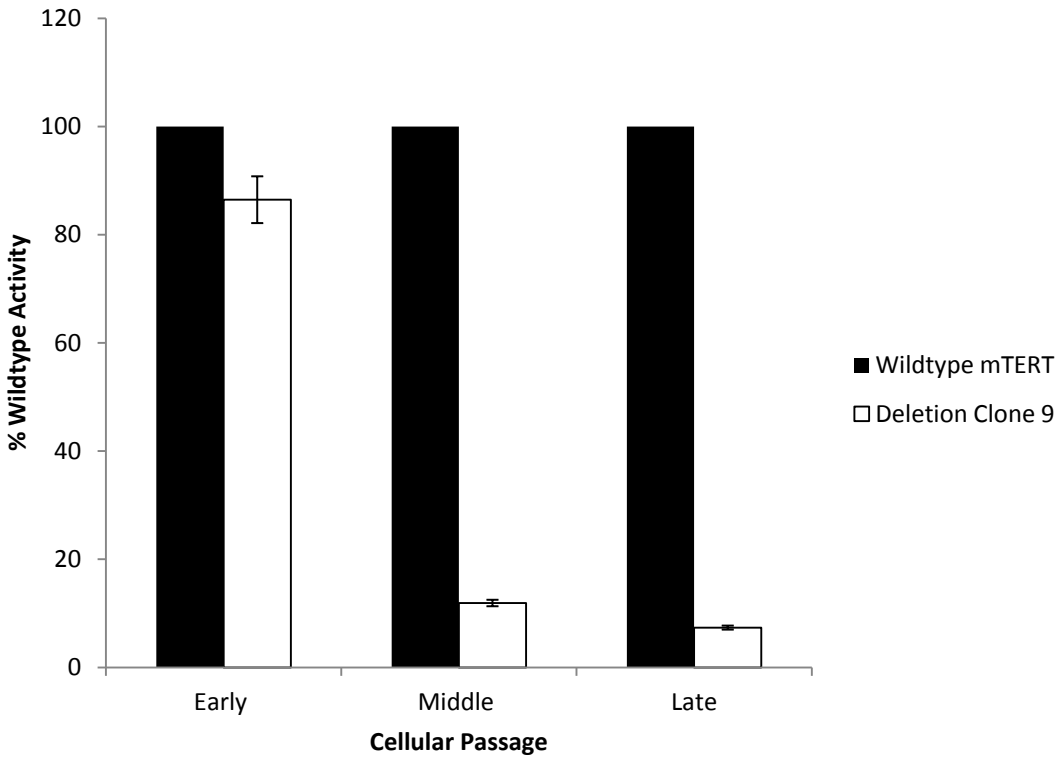


- Confirm levels by qPCR
- Confirm protein expression by Western analysis

Stable expression of the deletion variant (clone 9) leads to inhibition of telomerase with increasing population doublings



Stable expression of the deletion variant (clone 9) leads to inhibition of telomerase with increasing population doublings



Conclusions

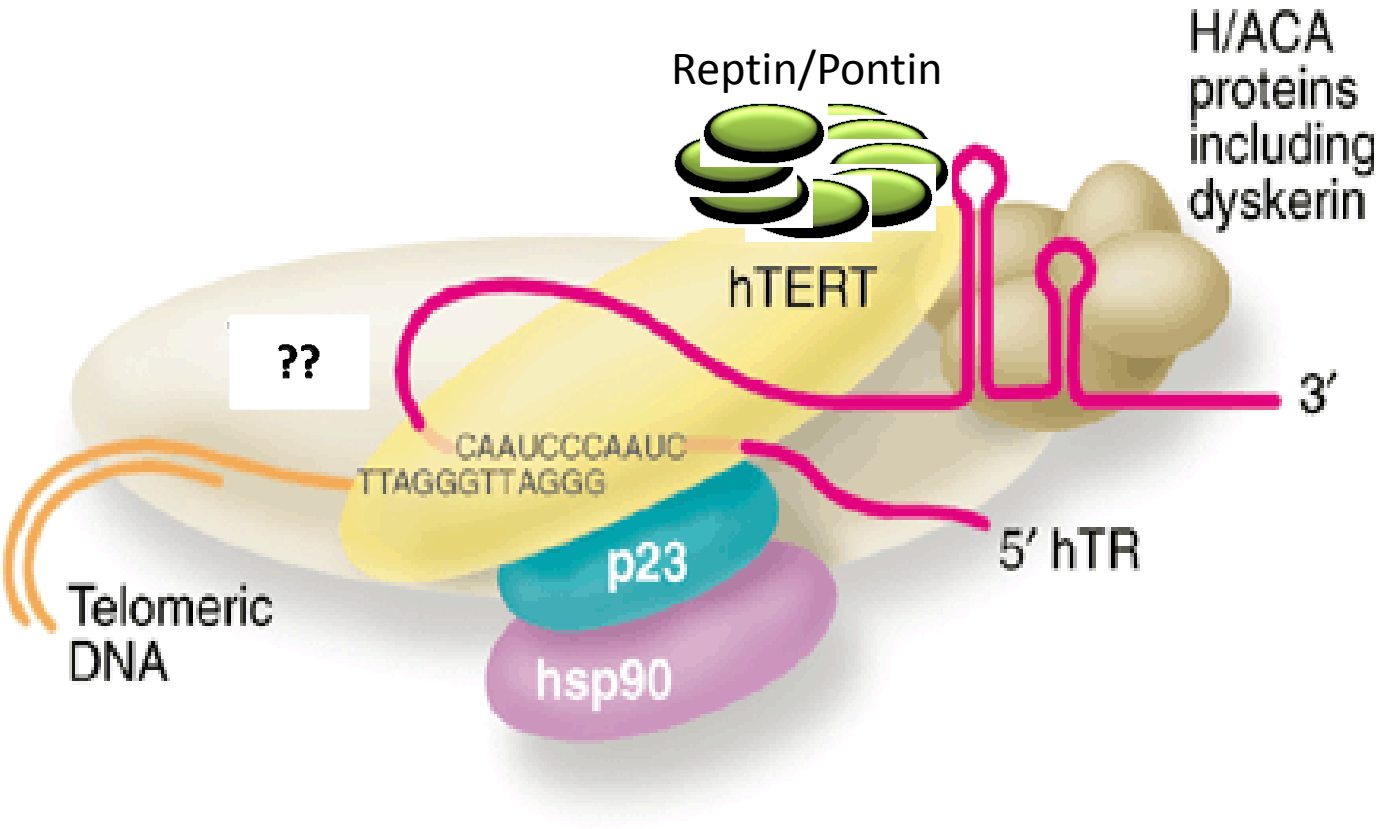
1. Five mTERT alternatively-spliced variants were identified by RT-PCR and sequencing.
2. The splicing patterns are different from rat and human (except for Dele6, which has also been identified in the human).
3. The variants were confirmed by RT-PCR using purified polyA+ mRNA from NIH 3T3.
4. In the *in-vitro* TRAP assay, the insertion variant reconstitutes telomerase activity while the deletion variant does not.
5. In vitro, while the insertion variant seems to have no inhibitory effects on wildtype mTERT, the deletion variant seems to be inhibiting telomerase activity and may have dominant negative effects on telomerase activity.
8. The insertion and deletion variants are present in different mouse cell lines and specific mouse tissues, shown through RT-PCR using variant-specific primers.
9. Deletion variant stable cells (clone 9) exhibit a noticeable growth defect and inhibition of telomerase activity with increasing population doublings

Perspectives

1. The potential negative-regulatory or dominant-negative function of the alternative-spliced deletion variants will continue to be assayed when expressed in telomerase-positive cell line, CB17 by assessing telomere length or function at various passages
2. Modulation of splicing in NIH 3T3 cells using anti-sense oligonucleotides and its consequences in gene expression, and telomerase activity and cell proliferation
3. Can alternatively-spliced mTERT variants function to reduce end-to-end fusions typically observed in late passage mTERT-/- ES cells by assessing signal-free ends

Identification of a Box C/D SnoRNP component as a human telomerase-associated protein

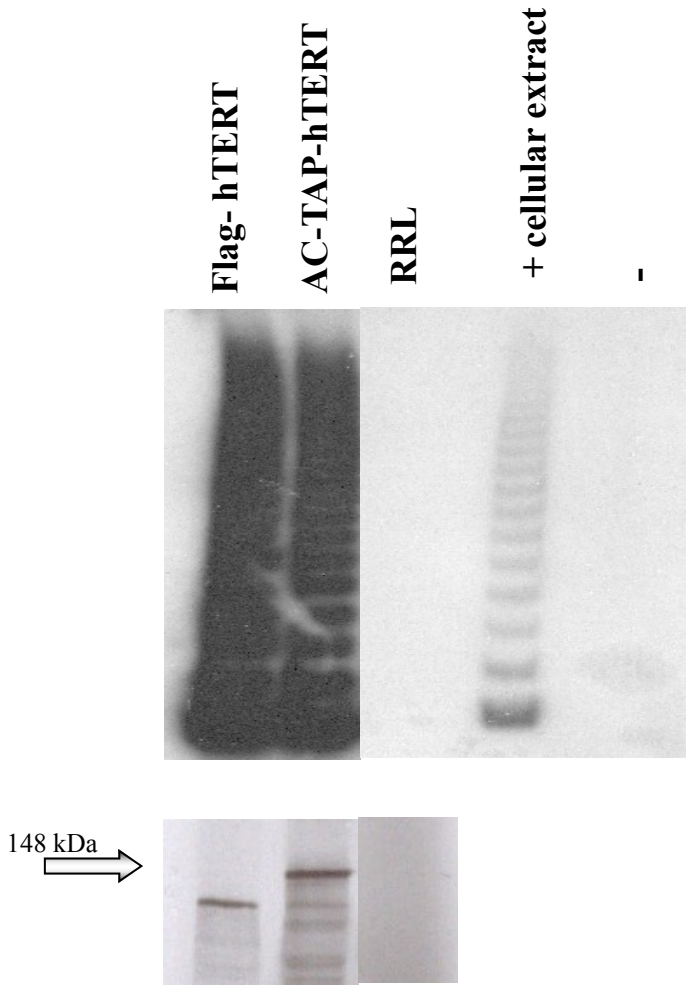
Telomerase-associated Proteins



Cohen et al, 2007 : dimer of hTERT, dyskerin and hTR (total of 1300kDa); Complex in the range of 500-1000 kDa (Schapp et al., 1998)

Shay et al, 1999

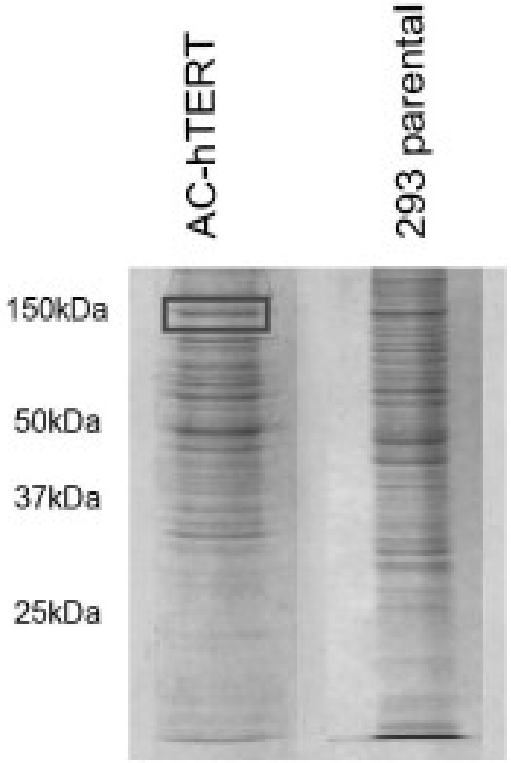
TAP-tagged hTERT reconstitutes an active enzyme in vitro



Created stably expressingTAP-tagged hTERT expressing Flp-In 293 cell line

Mass Spectrometry identifies NOP17 as a potential interacting protein

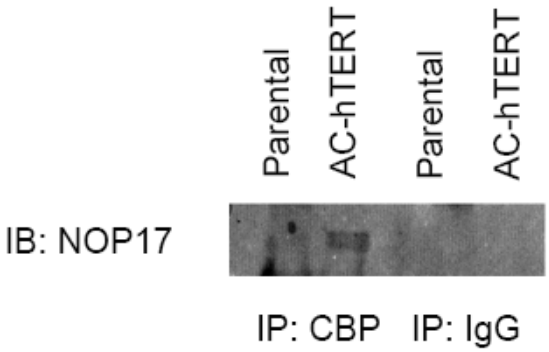
A

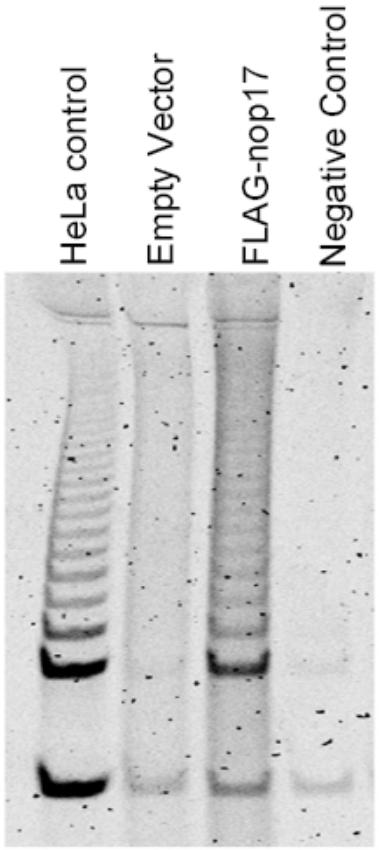


B

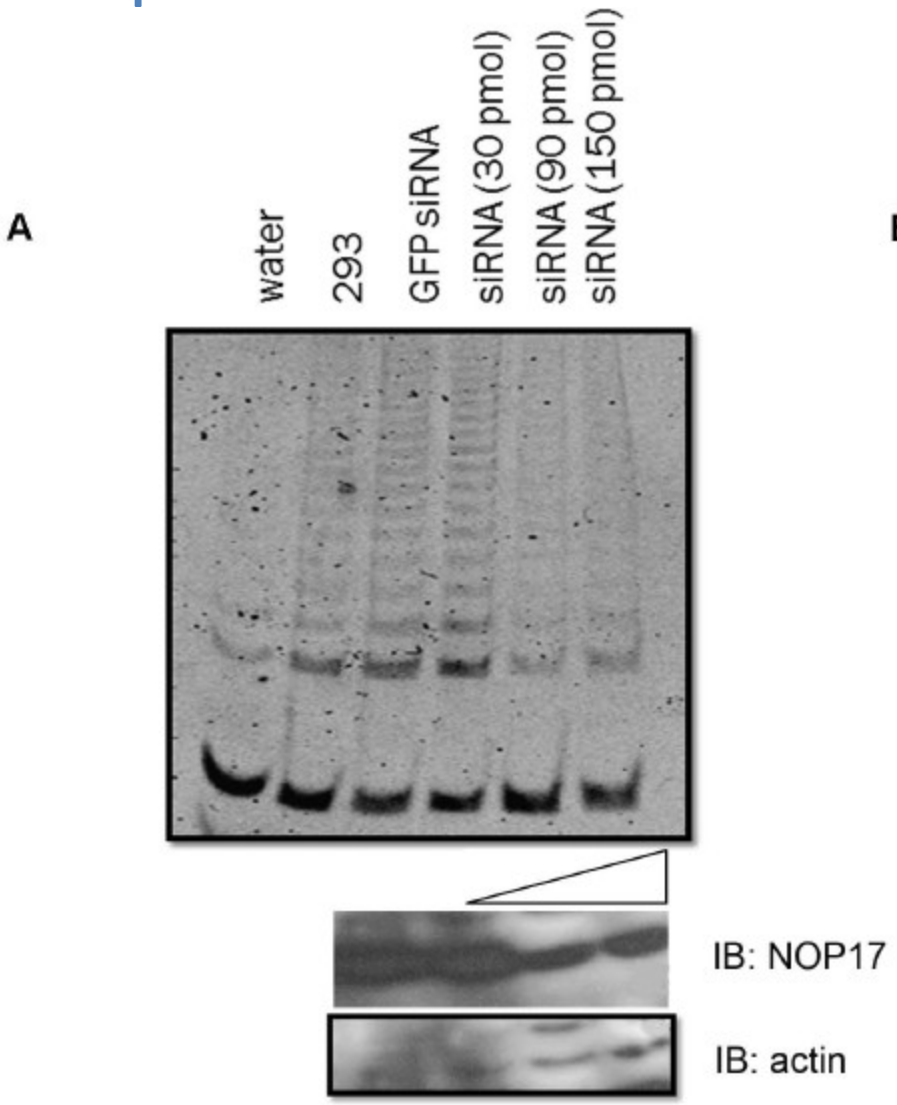
Protein	Size (kDa)	Unique Peptides	% sequence coverage
hTERT	127	15	19
Pontin	49	10	21
NOP17	32	6	33

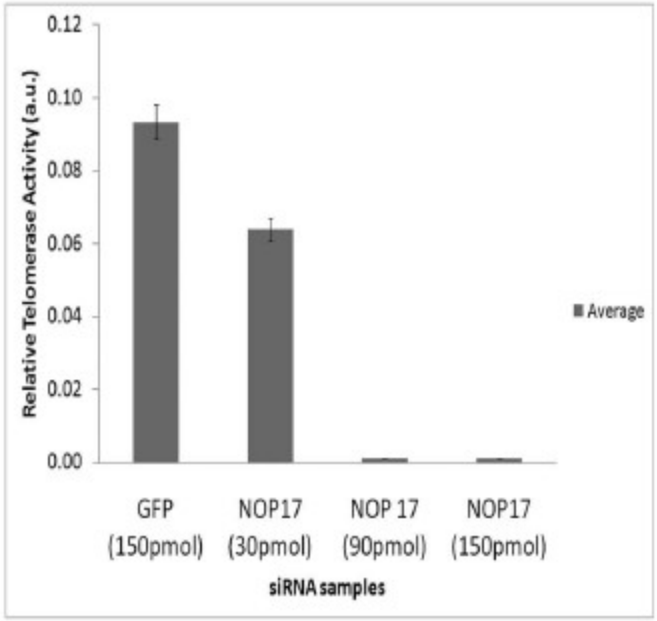
NOP17 :a telomerase interacting protein





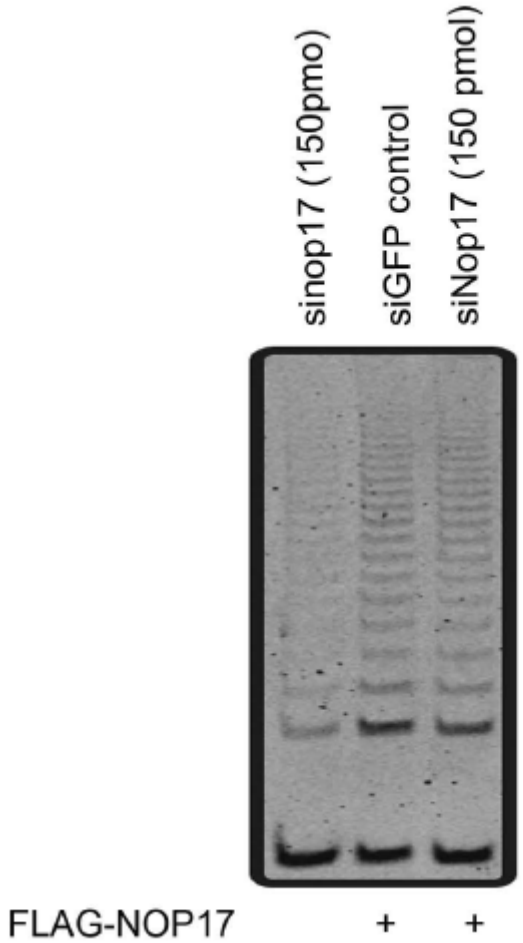
Depletion of NOP17 reduces telomerase activity

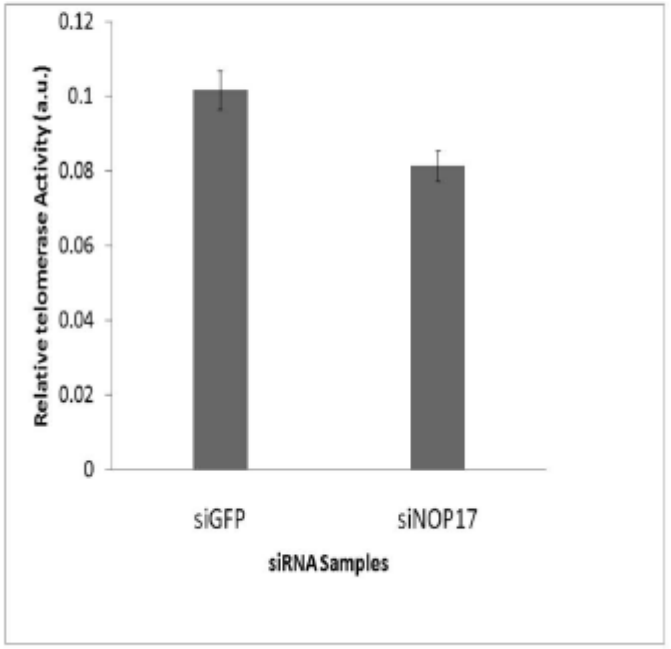




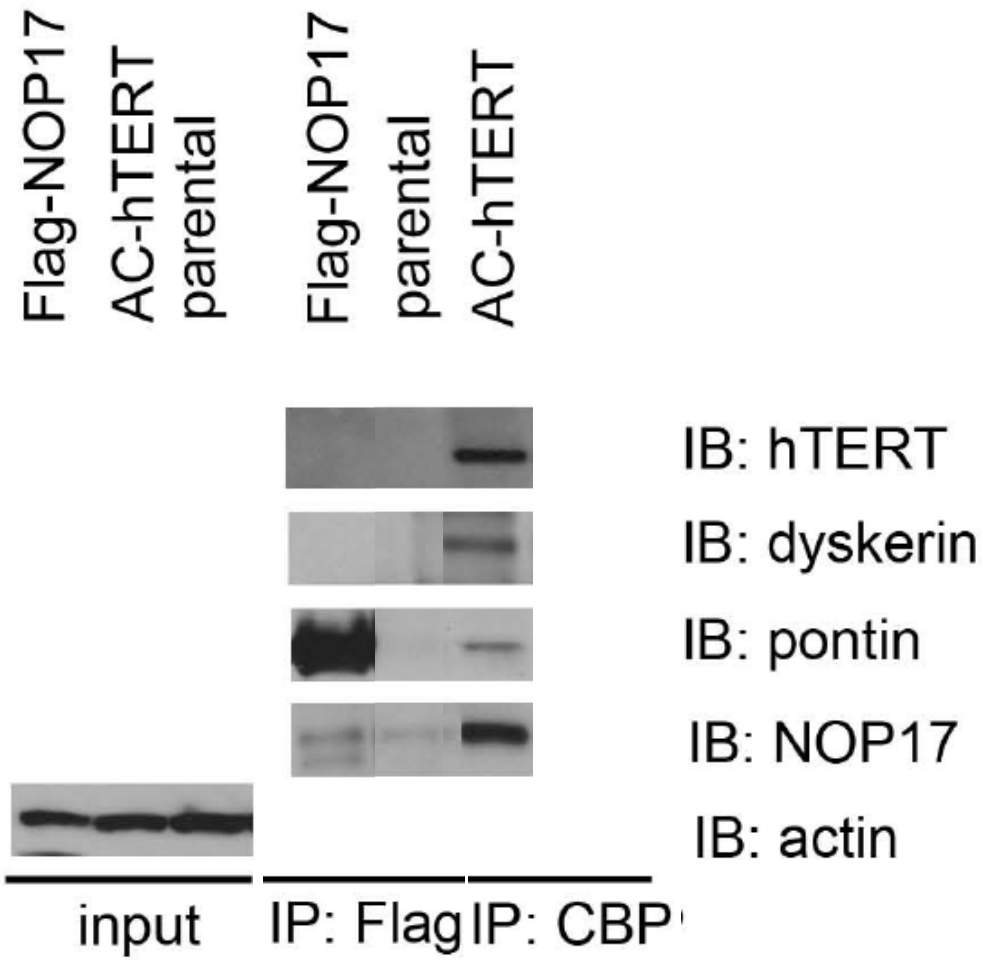
p = 0.017

NOP17 overexpression prevents the knockdown of telomerase activity

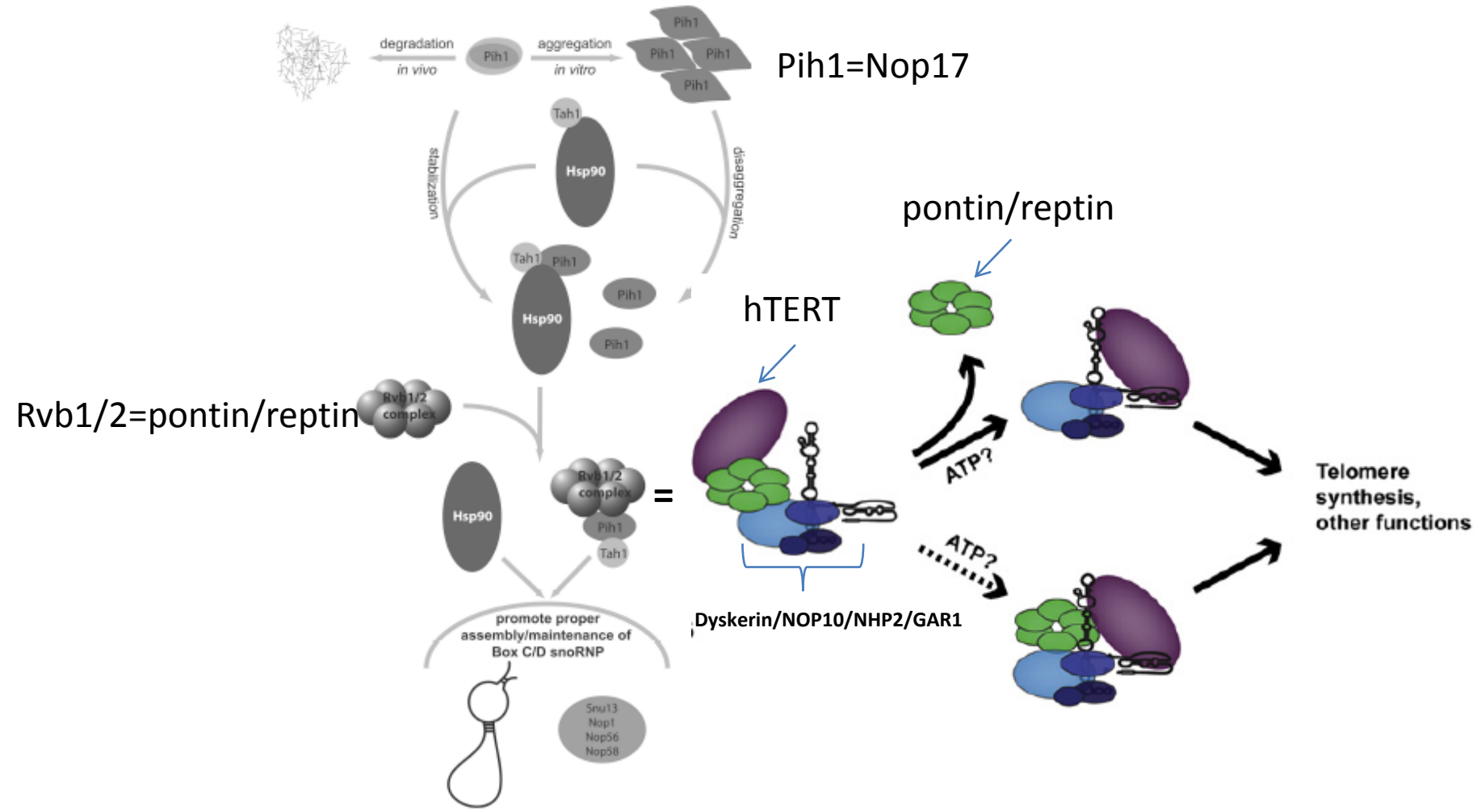




NOP17 forms a complex with pontin



Role for Nop17 in telomerase assembly?



Conclusions and Perspective

- NOP17 is a potential interacting protein of telomerase
- Immunoprecipitation against calmodulin binding peptide tagged hTERT confirms coimmunoprecipitates NOP17
- Immunoprecipitation of FLAG-NOP17 co-immunoprecipitates telomerase activity
- siRNA against NOP17 reduces telomerase activity
- Overexpression of NOP17 prevents knockdown of telomerase activity

- Assess if stable knockdown of NOP17 causes telomere shortening and reduced accumulation of hTR
- Assess if hTR, and various snoRNAs (H/ACA and C/D) co-immunoprecipitate with NOP17

Characterization of unique properties of telomerase that regulate telomere maintenance

Unique features of telomerase such as its repeat addition mechanism of DNA polymerization could be excellent specific targets

What are the determinants of processivity?

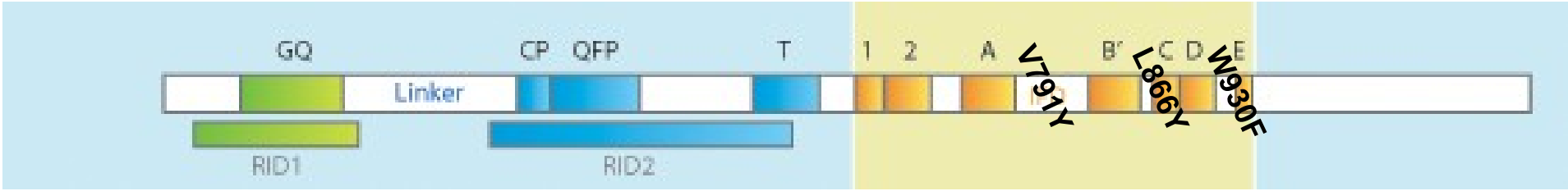
In vitro studies implicate TR, TERT, telomere and telomerase-interacting proteins (TPP1, Pot1), proofreading activities?

Does processivity *in vitro* correlate with telomere maintenance?

Emerging pharmacological and genetic evidence, primarily in yeast, suggests that telomerase processivity is a significant determinant of telomere length

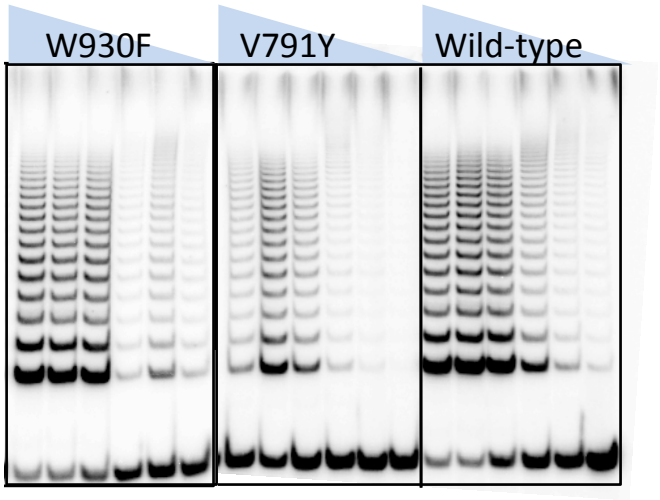
However, human telomerase is much more processive than yeast telomerase, and a fundamental unknown aspect of human telomerase biology is the contribution of enzyme processivity to telomere length maintenance and human cellular immortalization

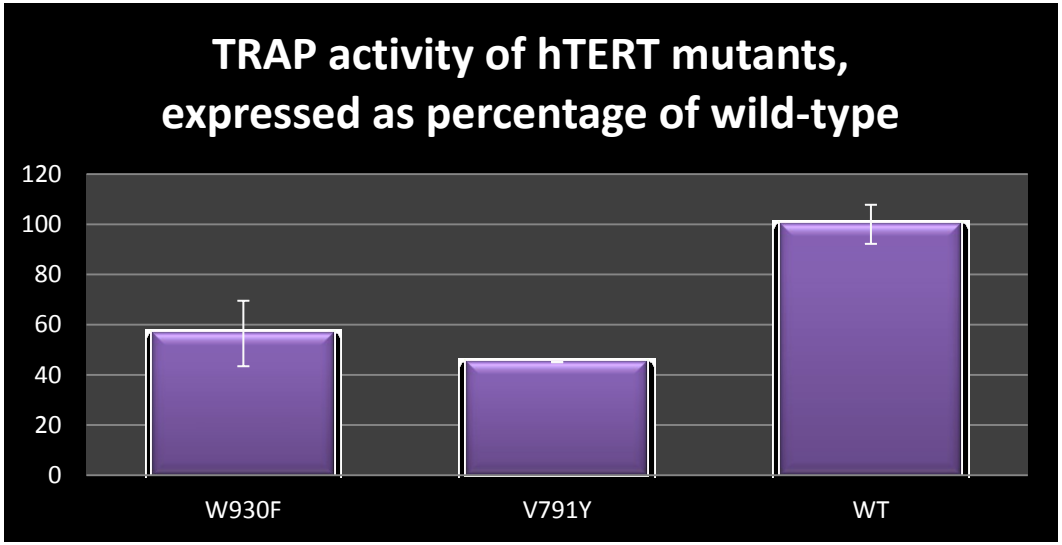
Residues predicted to be important for processivity



hTERT Mutant	Previous Mutation (TERT species)	Phenotype of TERT mutant	Effect on telomere length	Reference
V791Y	LYID589AAAA (Est2p)	Decreased processivity	Telomere shortening	Lue et al, 2003
W930F	FCA720WCG (Est2p)	Increased processivity	Telomere length increase	Peng et al, 2001
L866Y	L813Y (tTERT)	Increased processivity	N/A	Bryan et al, 2000

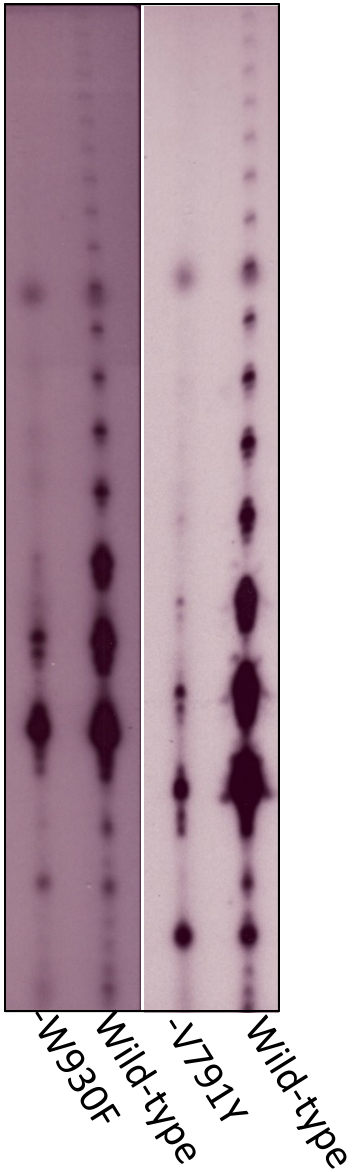
In vitro-reconstituted hTERT RT domain mutants -W930F and -V791Y are less active than wild-type



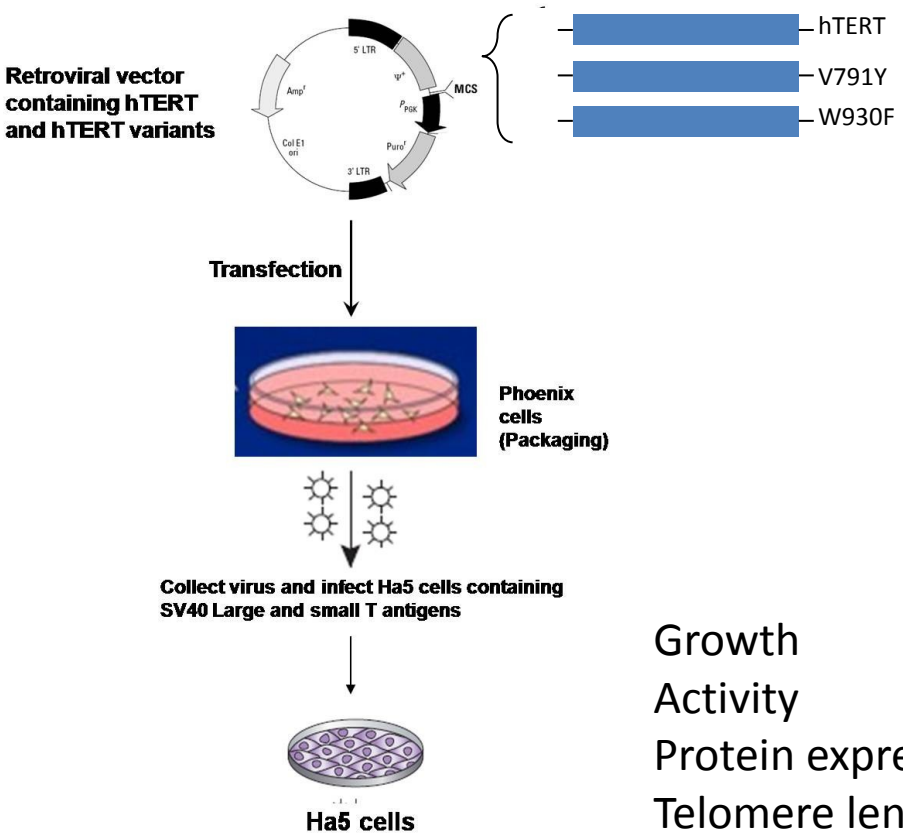


Processivity of *in vitro*-reconstituted hTERT-W930F and -V791Y enzymes are severely compromised

hTERT mutant	T2AG3 repeats
-W930F	3
-V791Y	4



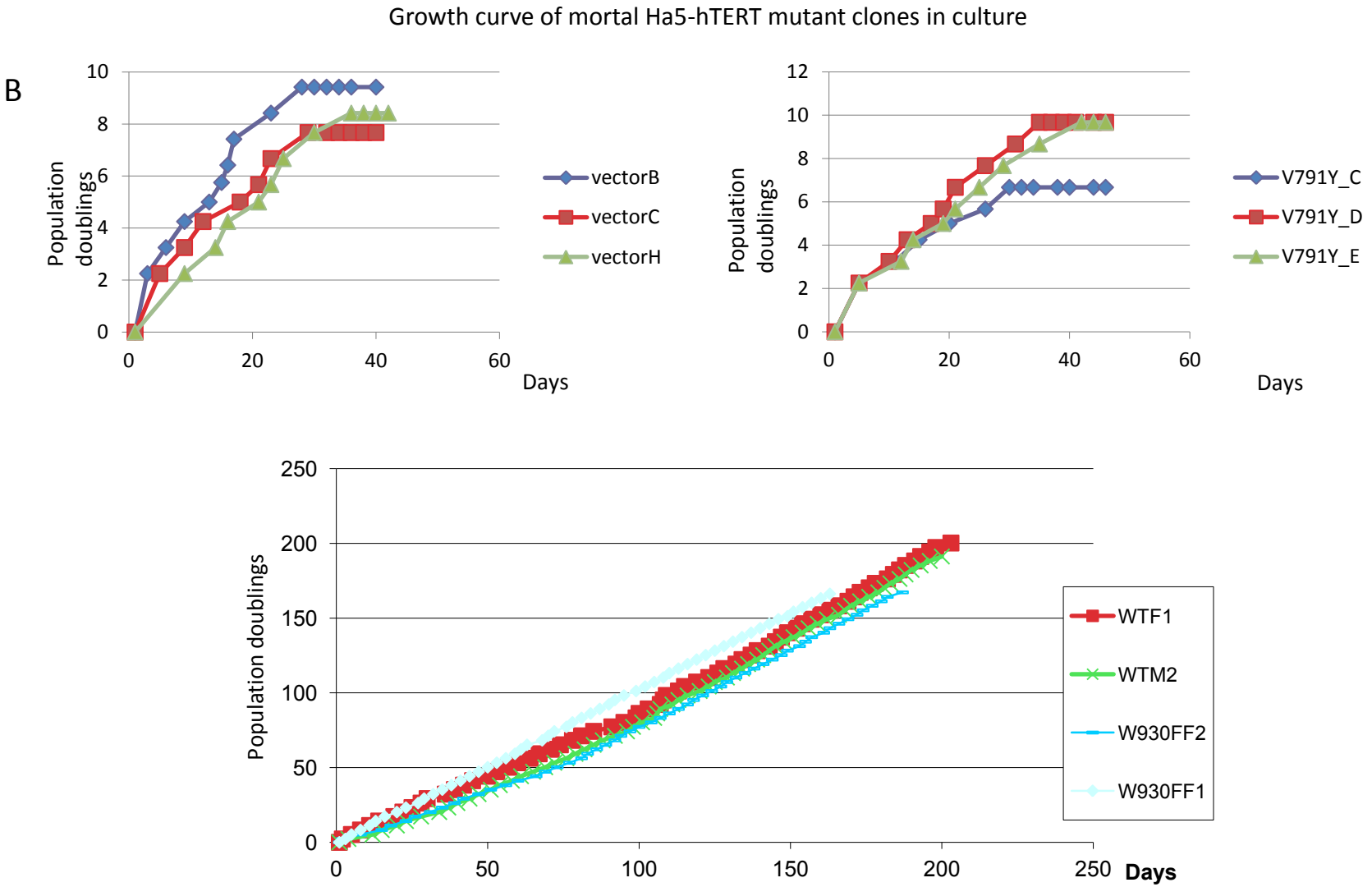
Mutant telomerases reconstituted in cells with limited lifespan



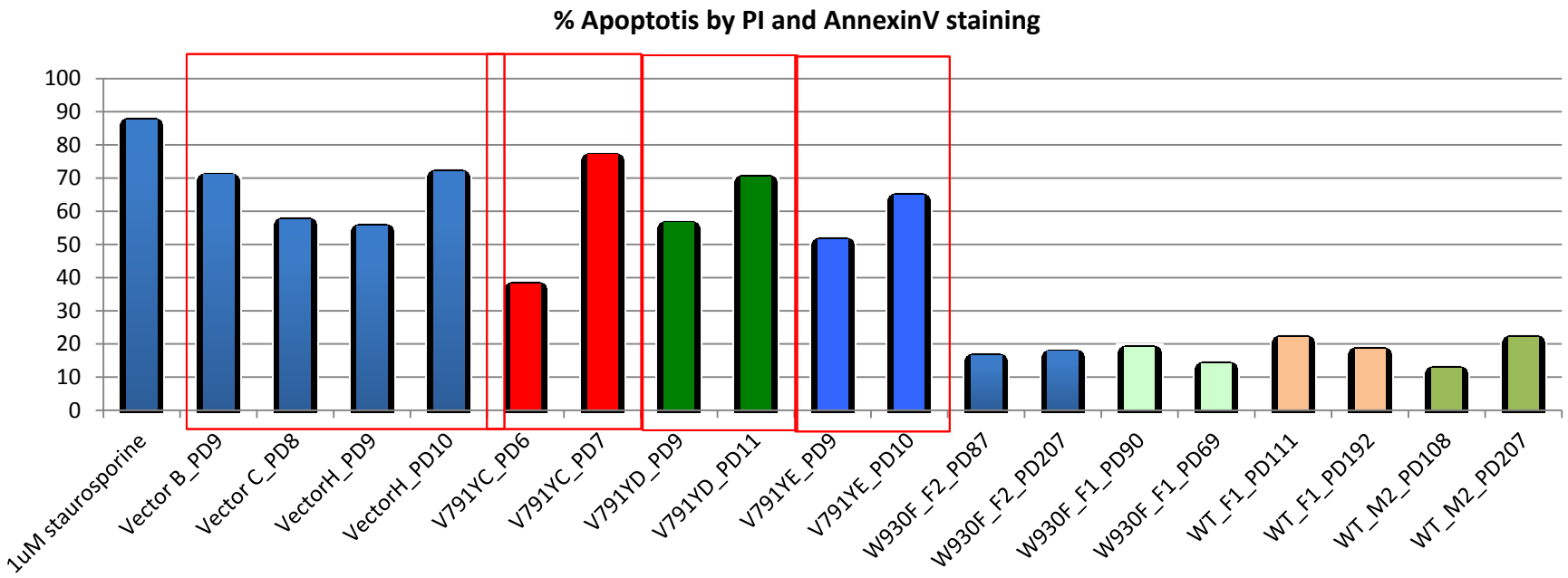
Growth
Activity
Protein expression
Telomere length

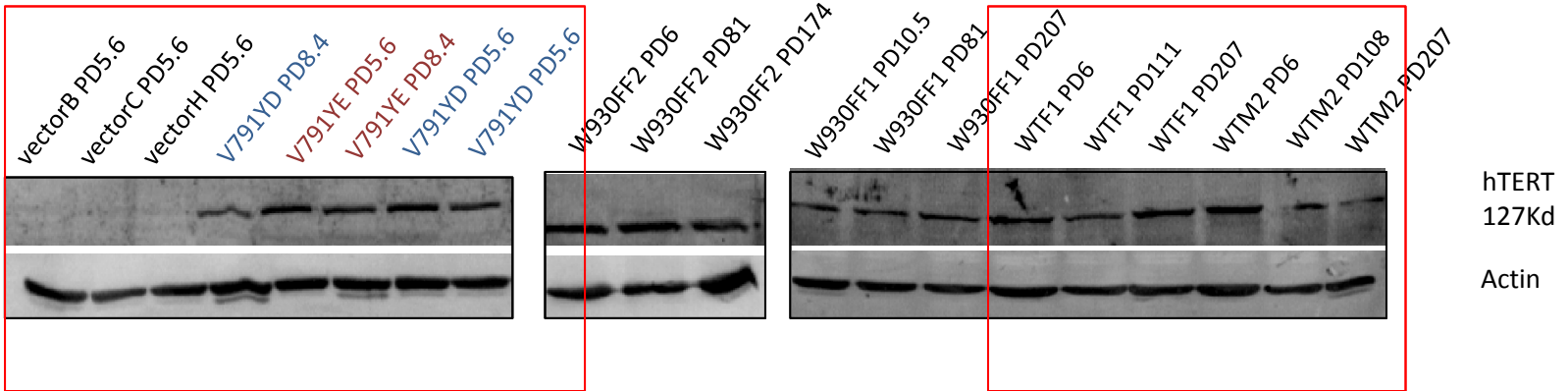
hTERT-/- hTR +/+
Fibroblasts expressing SV40 early region
Late passage, near crisis

Despite similar activities of *in vitro*-reconstituted enzymes, HA5 cells expressing hTERT-V791Y are unable to survive in culture, unlike HA5 cells expressing hTERT–W930F

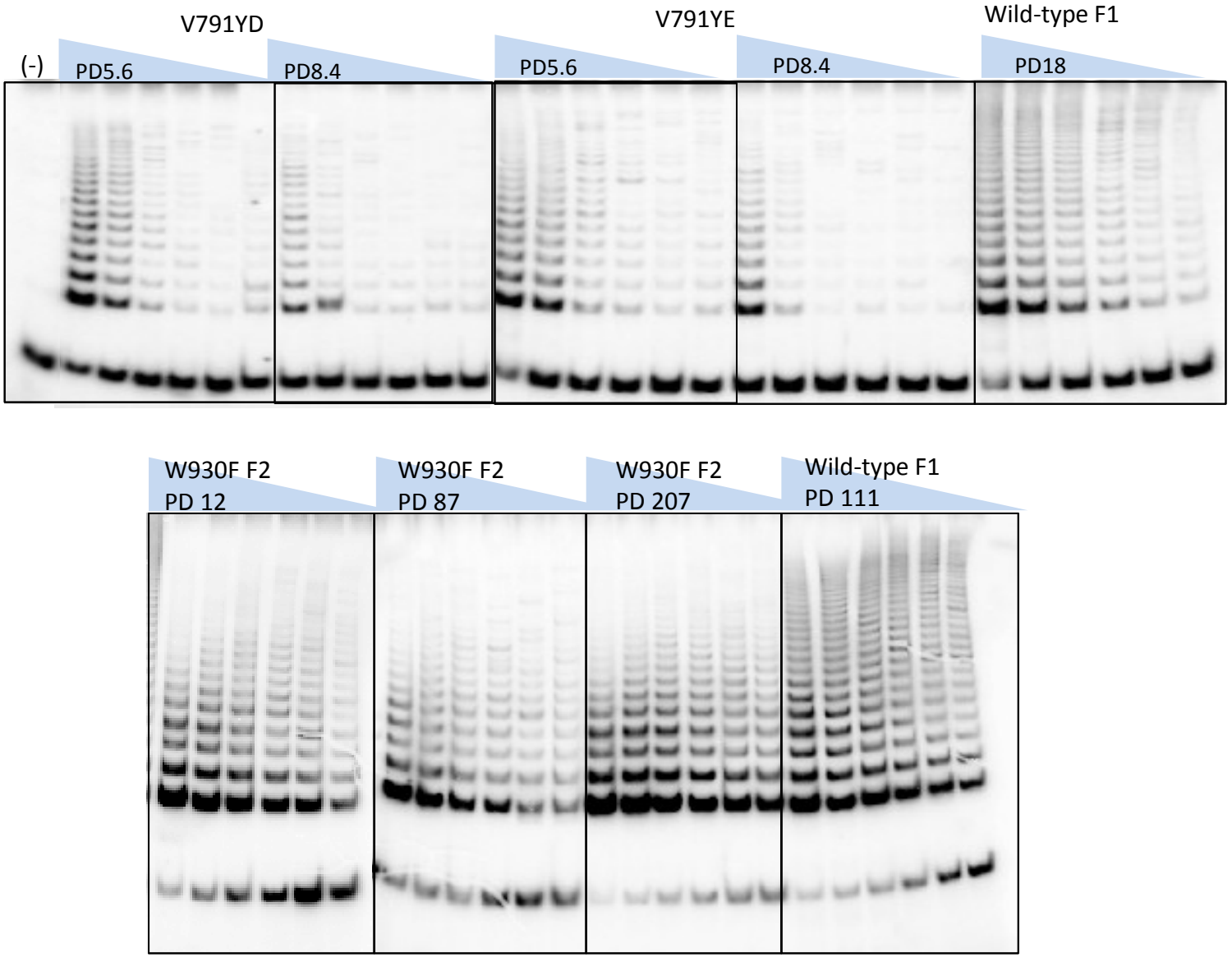


HA5 cells expressing hTERT-V791Y undergo apoptosis



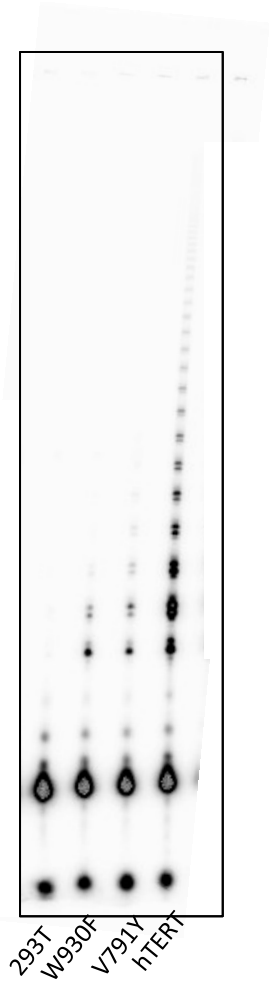


hTERT-V791Y and hTERT-W930F expressed in cells can reconstitute active enzymes



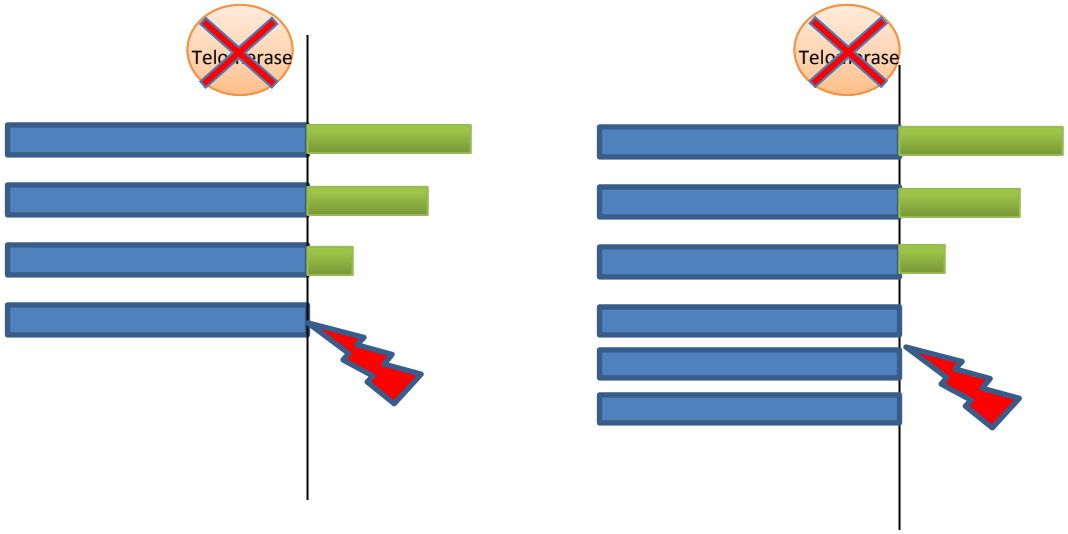
The processivity of mutant telomerase enzymes expressed in cells parallels the processivity of in vitro-reconstituted enzymes

Overexpress both hTERT and hTR components in 293T cells, collect extract and perform direct primer extension assay

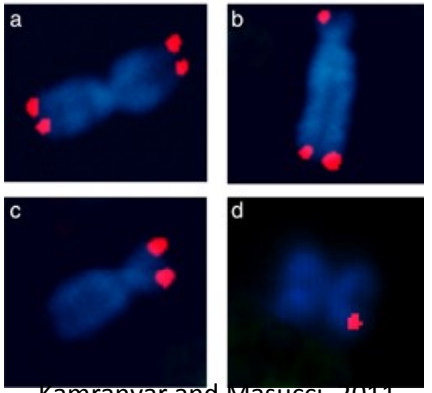


Critical telomere length

- It is disputed whether it is one critically short telomere or a subset of critically short telomeres that directs the cell towards senescence/cell death

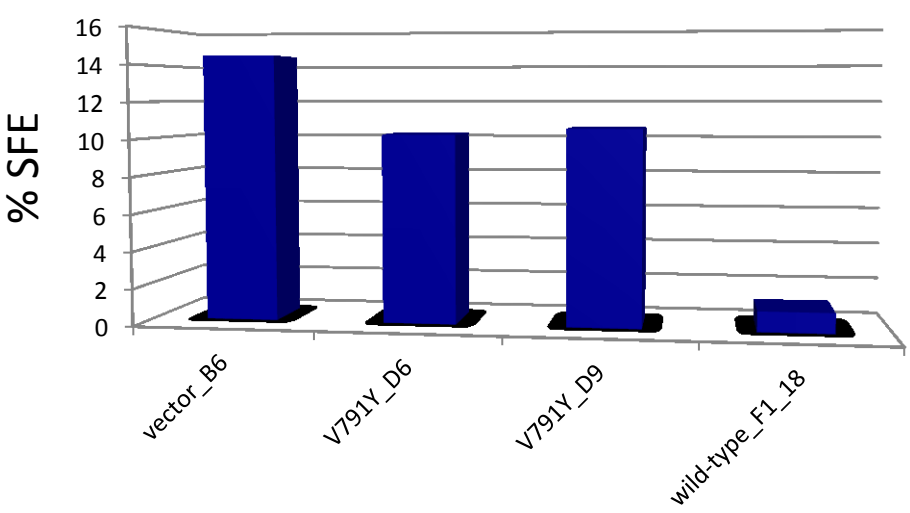


Critically short telomere: “SIGNAL FREE END” (SFE)



Kamranvar and Masucci, 2011

HA5 cells expressing hTERT-V791Y contain higher number of telomeric signal free ends than cells expressing hTERT–W930F



ˆ000
elomere
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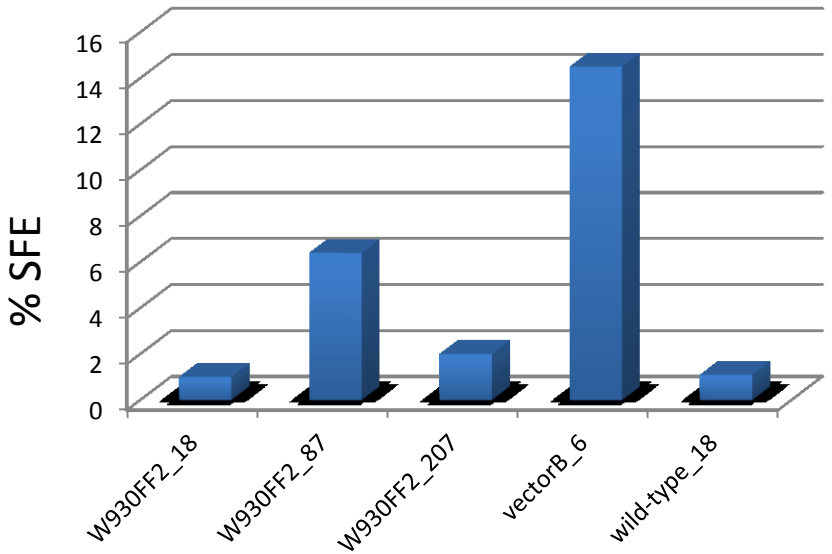
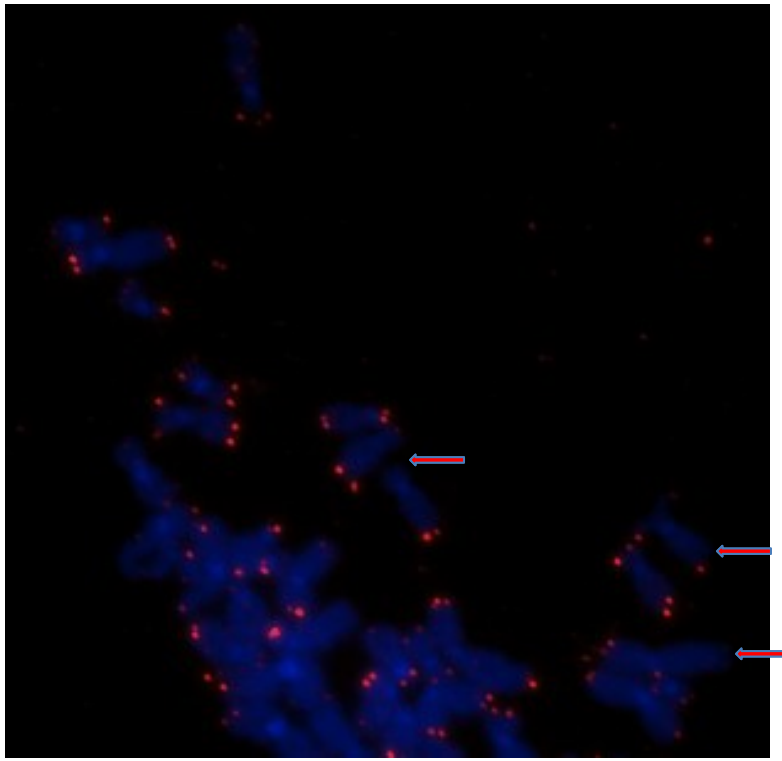


1000
telomere
ends



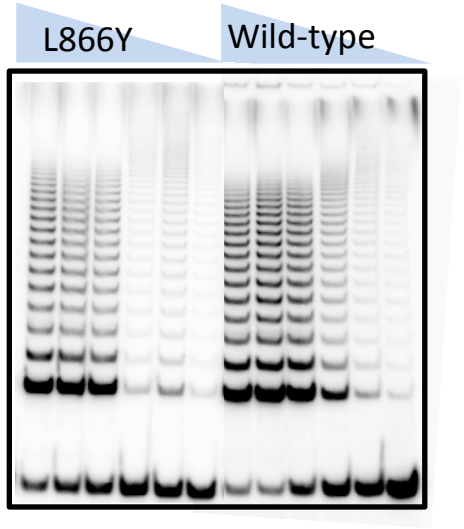
Possible identification of hTERT residues that regulate substrate utilization and/or recruitment to telomeres

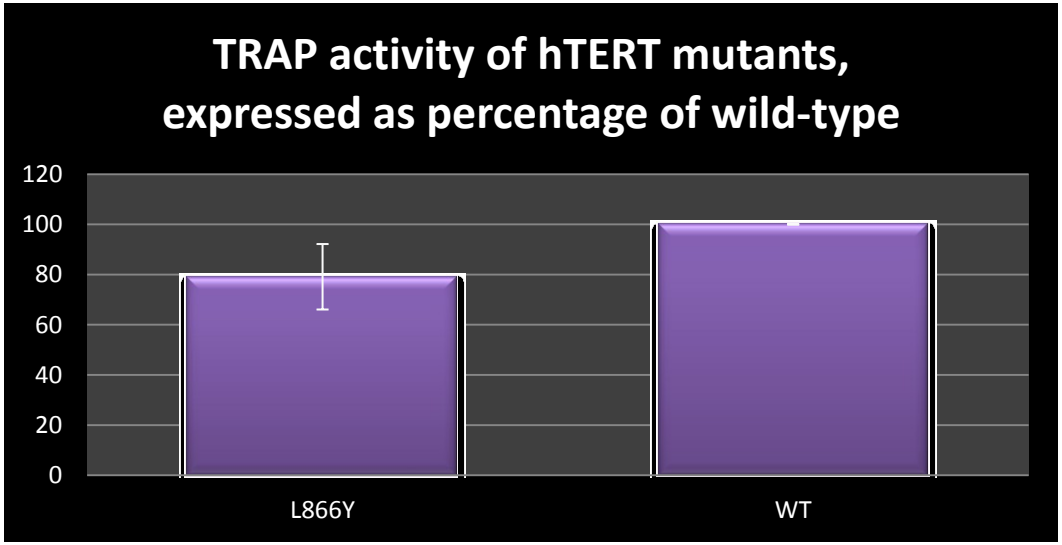
- Telomerase enzymes with similar activities and processivities function differently in telomere maintenance
- Activities and processivities of *in vitro*-reconstituted enzymes and enzymes expressed in cells are similar
- hTERT-W930F and -V791Y both accumulate SFEs however only -W930F is able to rescue those SFEs

Conclusion

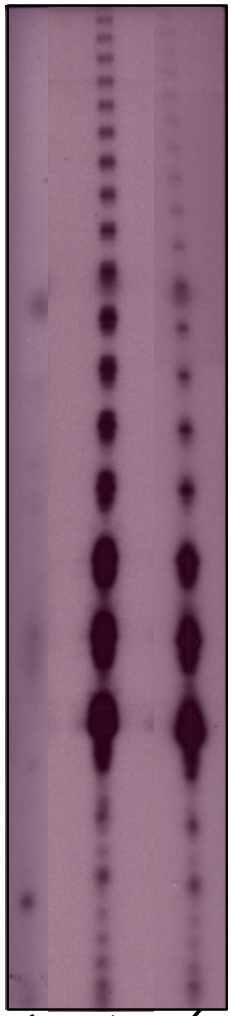
- ***We speculate that hTERT-W930F may more efficiently bind or elongate shorter substrates than hTERT-V791Y, or be more efficiently recruited to telomeres, allowing it to immortalize late passage HA5 cells with short telomeres***

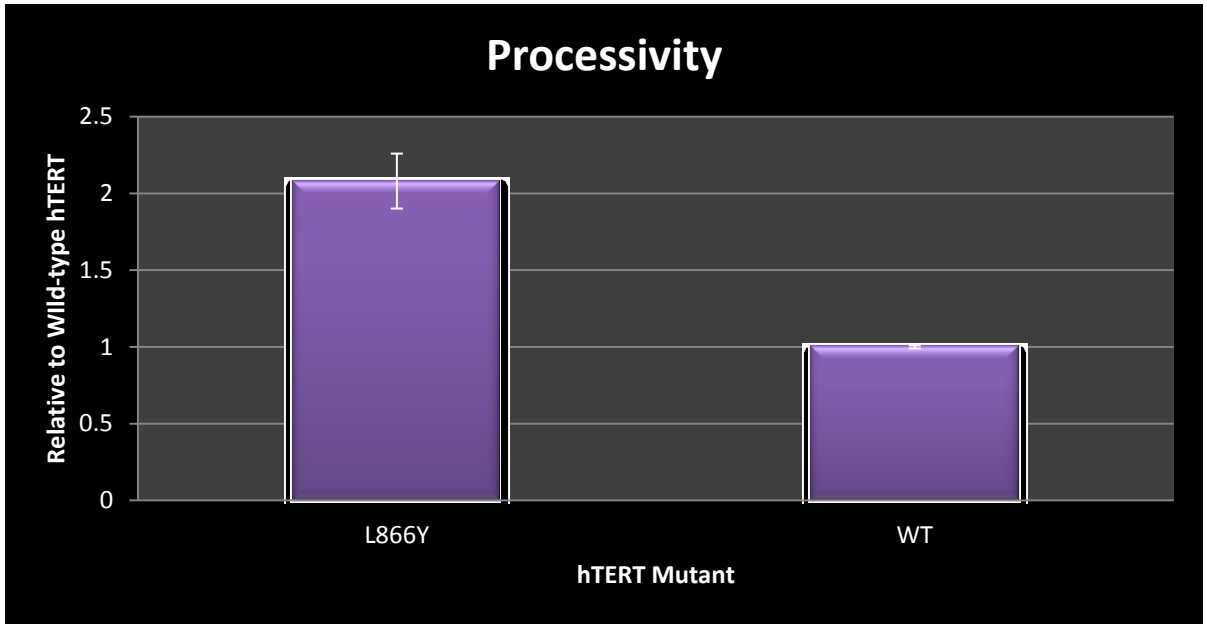
In vitro-expressed hTERT-L866Y reconstitutes similar levels of activity than wild-type enzyme



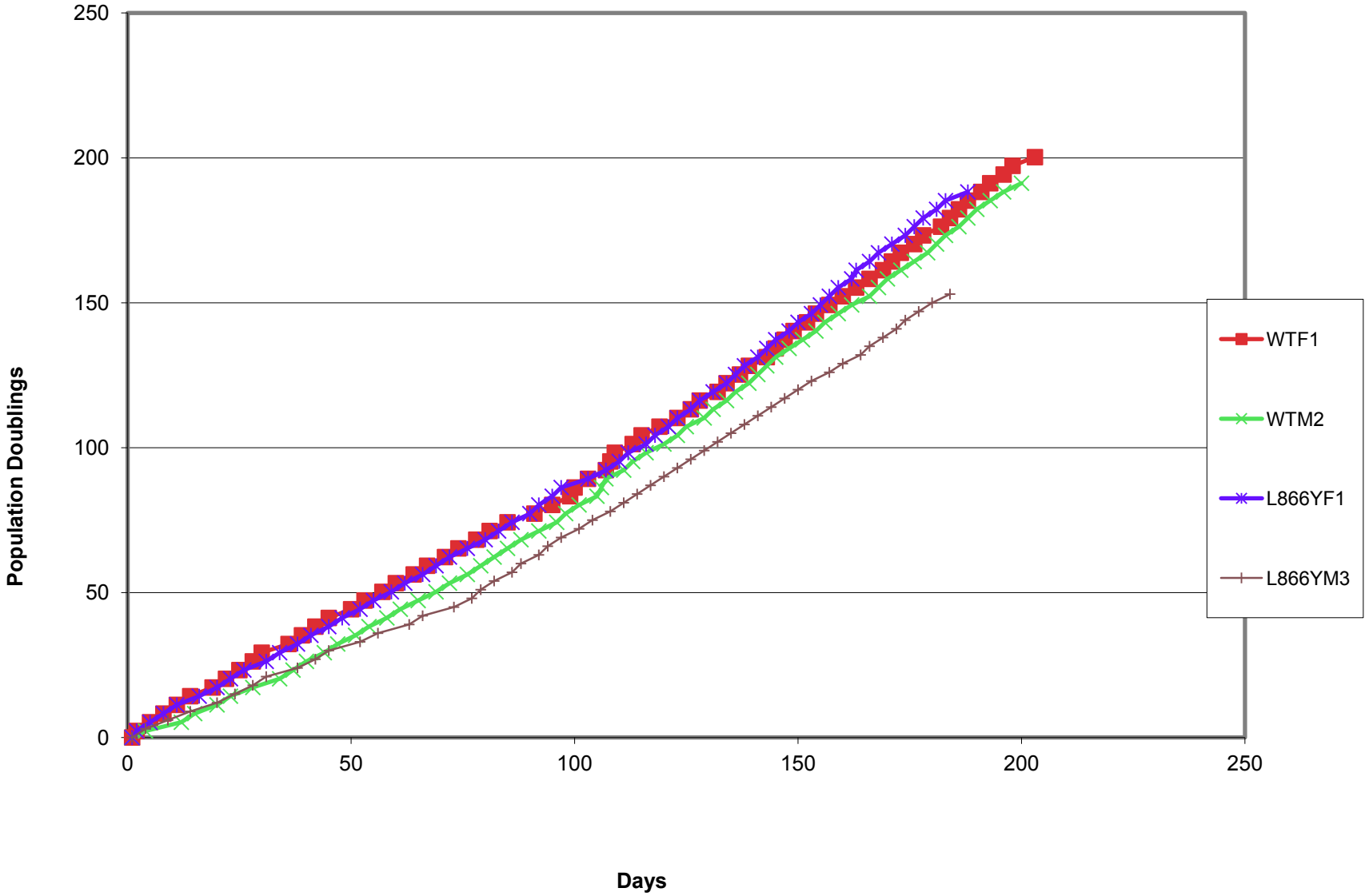


In vitro-expressed hTERT-L866Y displays increased processivity compared to wild-type enzyme

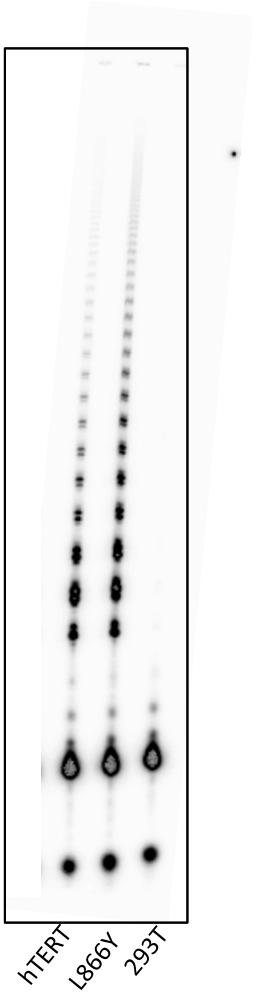


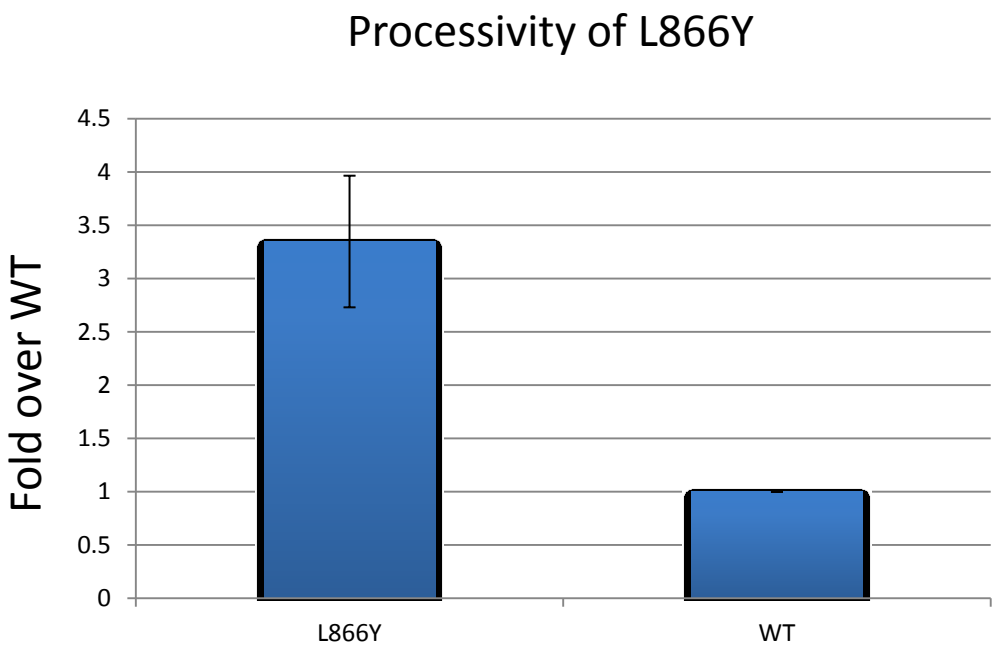


Growth rate of HA5-hTERT-L866Y is similar to HA5 expressing wild-type hTERT despite increased processivity



hTERT-L866Y enzyme extracted from cells is highly processive



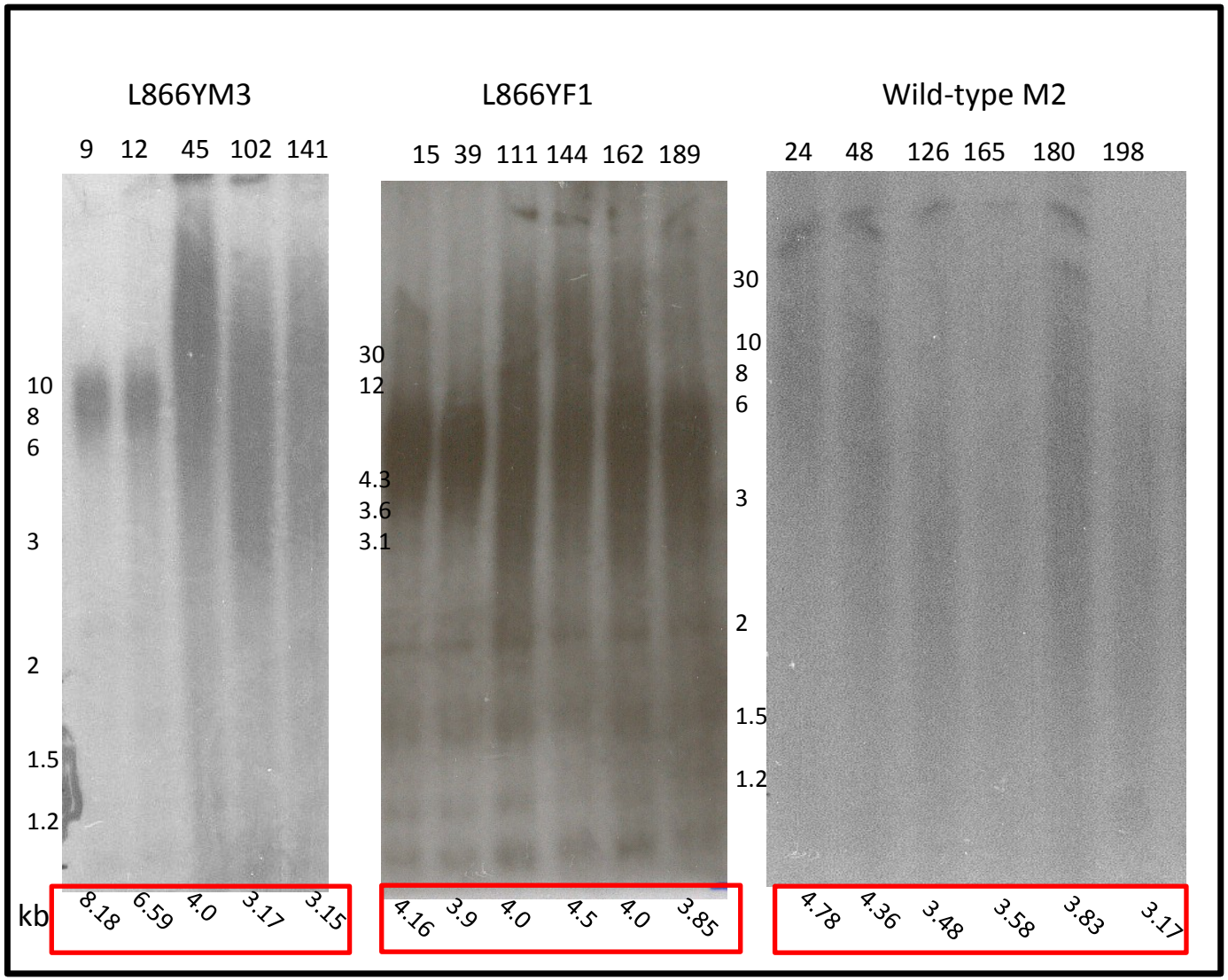


hTERT-L866Y-expressing clones display heterogenous telomere lengths

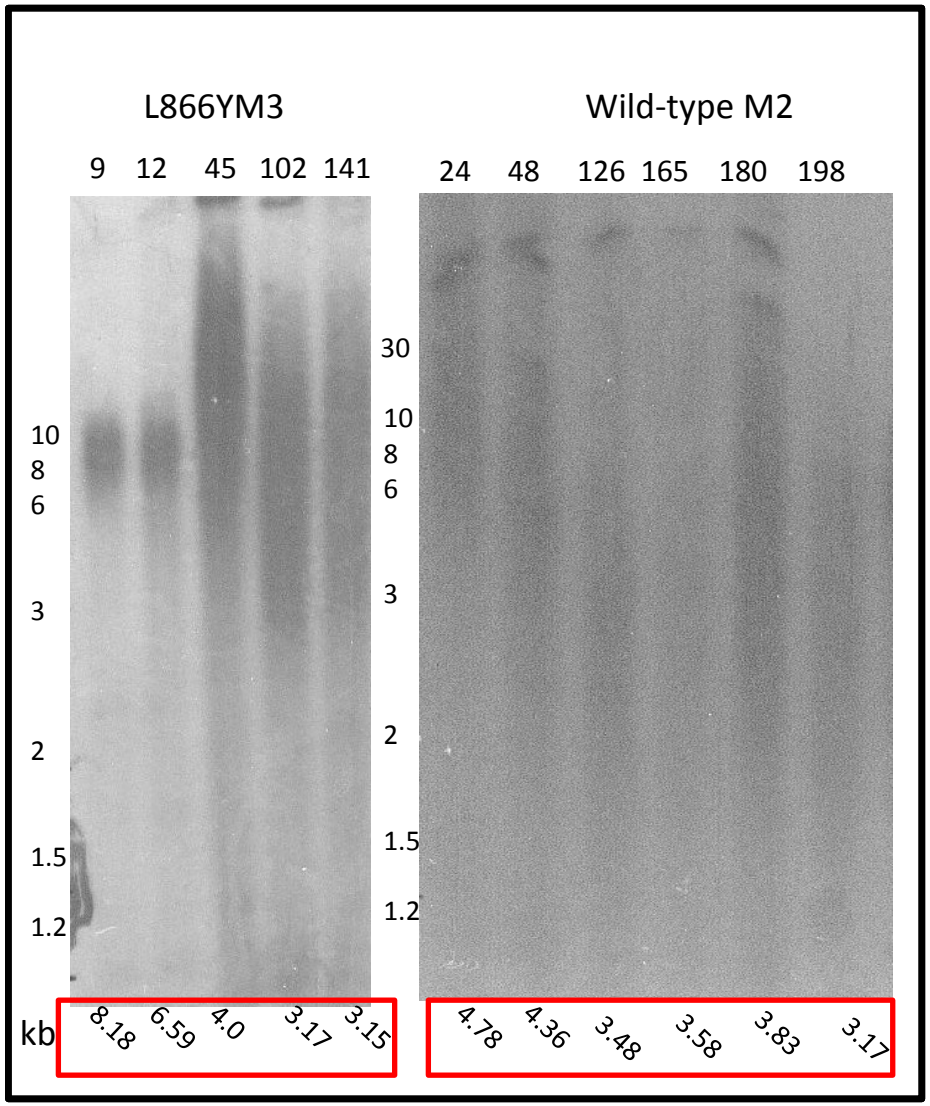
Digest with RsaI and HinfI

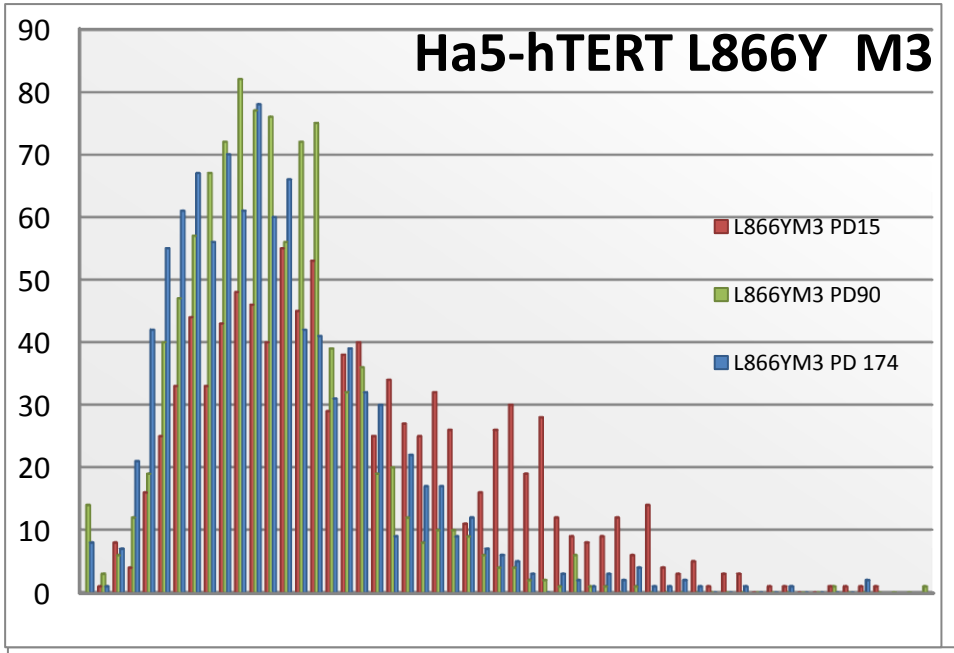
PFGE

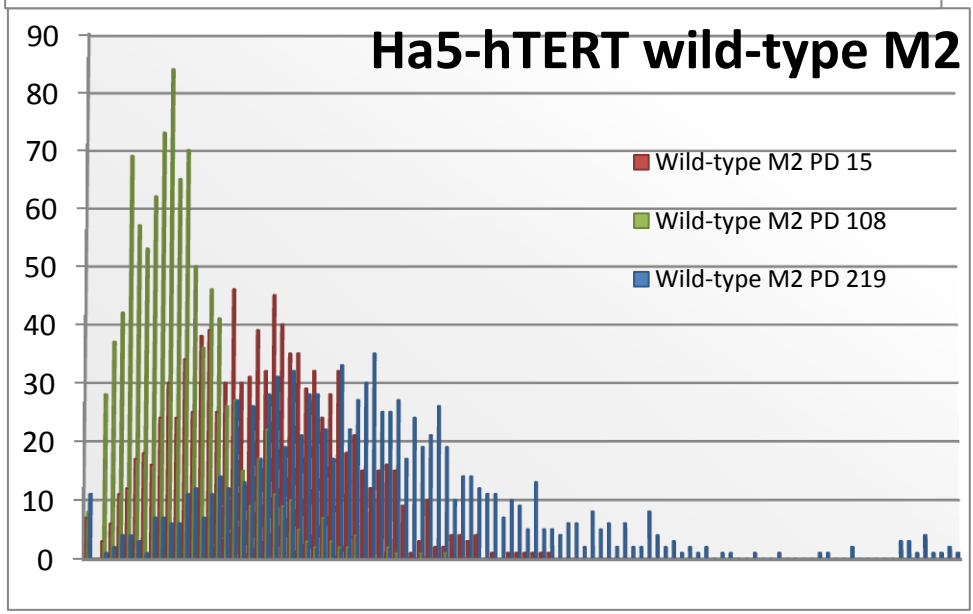
Probe with radiolabelled (C3TA2)4



hTERT-L866Y-expressing clones initially display an increase in telomere length followed by telomere length heterogeneity

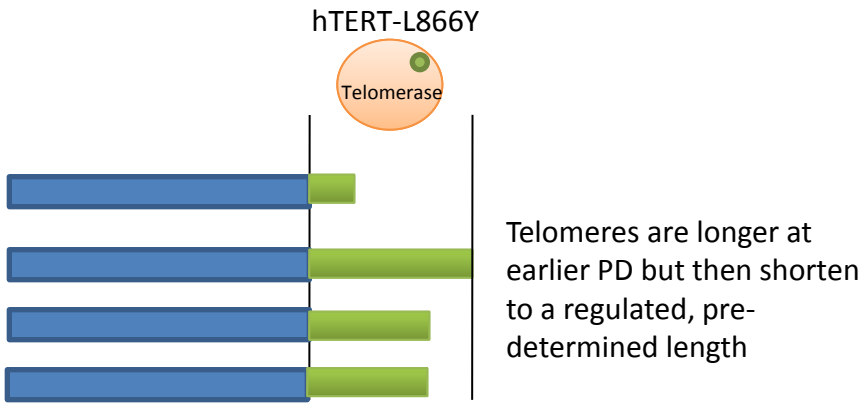






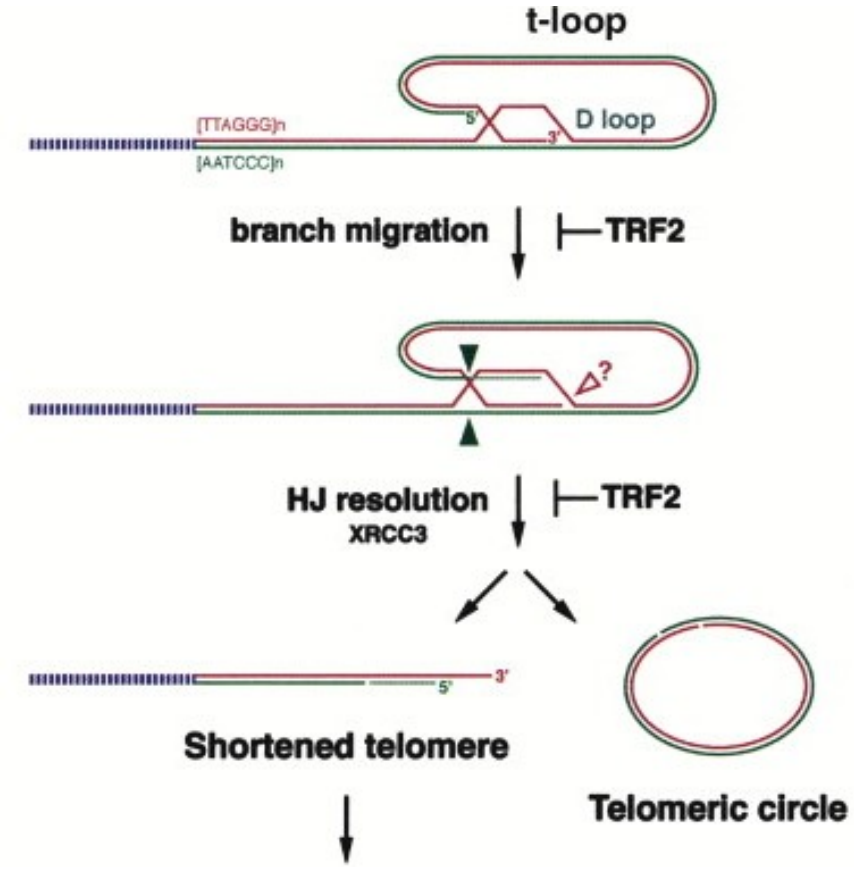
Negative regulation of telomere length by a telomeric DNA trimming mechanism

- Overexpression of both telomerase components in telomerase positive cancer cells results in increased telomere length that eventually reaches a plateau, accompanied by ALT characteristics such as telomere length heterogeneity and extrachromosomal telomeric repeat (ECTR) DNA in the form of T circles (Pickett et al, 2009)
- Not all characteristics of ALT were observed, including no telomere exchange events and no telomere dysfunction-induced foci
- Are we observing telomere trimming in HA5 cells expressing L866Y?



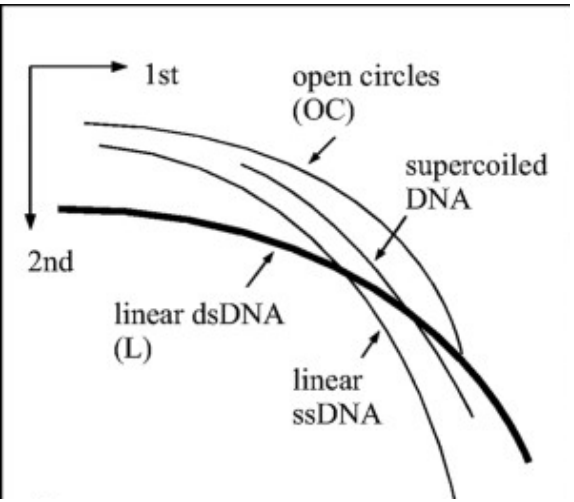
Telomeres are longer at earlier PD but then shorten to a regulated, pre-determined length

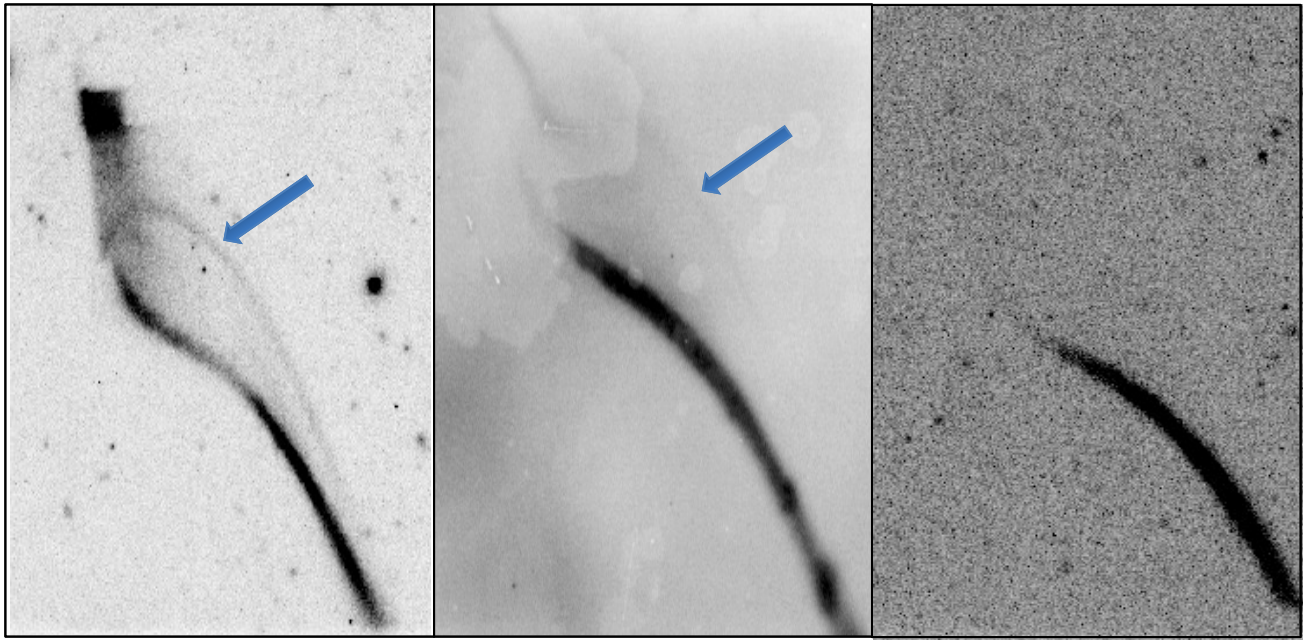
ECTR (T circle) formation



Wang et al, Cell, 2004

Presence of T-circles in late passage hTERT-L866Y-expressing cells suggest that trimming events are occurring





GM847 ALT cell

Ha5-hTERT-L866YF1
PD198

Ha5-hTERT
PD207

Regulation of telomere length and homeostasis by processivity

- L866Y reconstituted telomerase exhibits increased processivity
- Telomeres of hTERT-L866Y expressing cells are heterogeneous in length
- T-circles indicative of telomere trimming is observed in late passage L866Y

Conclusion

- ***We speculate that processivity is a regulator of telomere homeostasis***
- ***This would be the first report that trimming occurs physiologically in limited lifespan cells that require telomerase expression for cell survival versus in immortal telomerase-positive cells that overexpress both telomerase components***

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