Telomere Research Network Virtual Annual meeting December 3-4, 2020

Stacy S Drury, PI U24

Tulane University

New Orleans La, 70118

NIH NIEHS

NIH NIA

Logistics

- Zoom login provided via registration
- If you have troubles – use chat box DIRECT to Alyssa Lindrose
- Please mute yourself, but we will also be able to mute individuals.
- Feel free to stretch move around, dance, with or without your video on as virtual meetings are tough to keep everyone engaged
- KEY NOTE speakers (Shay/Herbig)- put questions in chat
- Other presentations- raise hand and we will unmute you

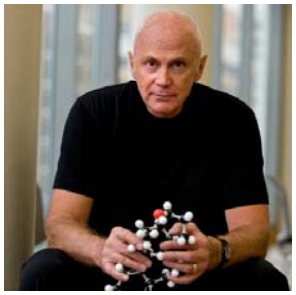
TELOMERE RESEARCH NETWORK

Telomere Research Network

U24


Drury


Epel


McLachlan


Verhulst


Simmons


Nielsen


Guo

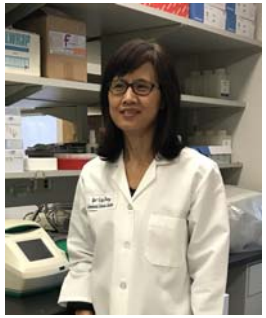

Heacock

U01s


Aviv


Lin


Shalev


Zheng

External Advisory Committee


Crimmins


Entringer


Shay


Eisenberg


Nettle


Belsky

TELOMERE RESEARCH NETWORK

Telomere Research Network: Background and NIH Goals

Michelle Heacock, NIEHS

Lis Nielsen, NIA

On behalf of the NIH Team

Molecular footprints/sentinels of stress and or exposures and resilience for understanding disease risk

Shorter

- Increased risk for some cancers
- Genetic susceptibility – telomere syndromes (e.g., lung diseases and cancers)
- Cadmium exposure
- Hyperoxia conditions
- Psychosocial Stress

Longer

- Increased risk for other cancers
- Protective for CVD
- Arsenic exposure
- PCB exposure

Telomere length

Haycock et al. 2017; Armanios & Blackburn 2012; Zota et al. 2015; Mitro et al. 2016; Kurz et al. 2004; Schutte et al. 2016

2017 NIEHS/NIA Workshop

Sessions: Psychosocial stress, Environmental exposures, Telomere length measurements, Combining markers, and Genetic susceptibility

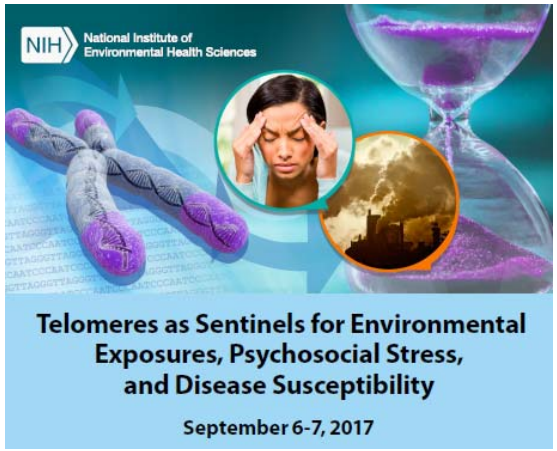
Basic researchers & population health researchers brought together for first time

Workshop Objectives:

- To explore current and future possibilities for using the telomere as a potential biomarker of environmental and stress exposure or disease susceptibility
- To discuss the tractability of using telomeres as a proxy/indicator of genomic damage
- To consider the themes outlined above in the context of tissue-specific effects, to identify which cells should, or can be used as proxy.
- Identify how interrogation of telomere status can currently enhance epidemiological studies and the potential for further research.
- Develop a set of recommendations for moving forward with telomere measurements in population-based studies, and identification of short- and long-term research needs.

Final report https://www.niehs.nih.gov/about/events/pastmtg/2017/telomeres/telomere_meeting_report_508.pdf

Workshop Recommendations:



Need for NIH stimulus to:

- Develop consensus guidelines
  - Sample collection, storage, data analysis, reporting requirements
- Conduct a robust methods comparison study
  - Compare assays, variability, foundation for guidelines
- Produce and provide standard reference samples
  - Calibrate telomere length measurements

Need for a coordinated effort to foster interdisciplinary collaborations

- Encourage more research on telomere length dynamics
- Focus on early life determinants
- Develop better measures of stress exposure
- Support an effort to collect unpublished knowledge on best practices

Key challenge, concerns with telomere length measurements

Department of Health and Human Services
Part 1. Overview Information

Participating Organization(s)	National Institutes of Health (NIH)
Components of Participating Organizations	National Institute on Aging (NIA) National Institute of Environmental Health Sciences (NIEHS)
Funding Opportunity Title	Telomeres as Sentinels of Environmental Exposures, Psychosocial Stress, and Disease Susceptibility: A Methods Comparison Study (U01 Clinical Trial Optional)
Activity Code	U01 Research Project – Cooperative Agreements
Announcement Type	New
Related Notices	• **November 26, 2018** - NIH & … Research Grant Applications. • October 10, 2018 - Notice of …
Funding Opportunity Announcement (FOA) Number	RFA-AG-19-023
Companion Funding Opportunity	RFA-AG-19-022, U24 Resource-Rel…

U01s:
Conduct a robust methods comparison study
Develop consensus guidelines

U24:
Support methods comparison study
Foster interdisciplinary collaborations
Encourage research
Share best practices

Department of Health and Human Services
Part 1. Overview Information

Participating Organization(s)	National Institutes of Health (NIH)
Components of Participating Organizations	National Institute on Aging (NIA) National Institute of Environmental Health Sciences (NIEHS)
Funding Opportunity Title	Research Network on Telomeres as Sentinels of Environmental Exposures, Psychosocial Stress, and Disease Susceptibility (U24 Clinical Trial Not Allowed)
Activity Code	U24 Resource-Related Research Projects – Cooperative Agreements
Announcement Type	New
Related Notices	• **November 26, 2018** - NIH & AHRQ Announce Upcoming Updates to Application Instructions and Review Criteria for Research Grant Applications. See Notice NOT-OD-18-228.
Funding Opportunity Announcement (FOA) Number	RFA-AG-19-022
Companion Funding Opportunity	RFA-AG-19-023, U01 Research Project – Cooperative Agreements

Methods Collaboratory:
A Linked Set of Cooperative Agreements

Collaboration among U01s, U24, and NIH staff

Grant Type	PI	Institution	Program Officer	Project Scientist
U01	Abraham Aviv	Rutgers	Max Guo	Michelle Heacock
U01	Jue Lin	UCSF	Max Guo	Michelle Heacock
U01	Idan Shalev	Penn State	Michelle Heacock	Max Guo
U01	Ling Zhang	George Washington University	Michelle Heacock	Max Guo
U24	Stacy Drury	Tulane University	Janine Simmons, Lis Nielsen	Max Guo, Michelle Heacock

TELOMERE RESEARCH NETWORK

Multiple Roles & Responsibilities Across the Network

Methods Comparison Project: U01 award funded under RFA-AG-19-023 working on the Methods Comparison Study.

Telomere Research Network (TRN): The U24 award funded under RFA-AG-19-022 and all participants in activities supported by the U24. TRN is expected to expand to engage and include other investigators in the broader field over time, through participation in workshops, pilot research programs, and other activities.

Steering Committee: The main governing board of the Telomere Research Network comprised of PDs/PIs and NIH Project Scientists from each U01 award and the U24 award as well as additional investigators and NIH staff as appropriate. Addresses issues that span the TRN and all Methods Comparison Projects, including providing input into the processes of the projects, and assisting in dissemination of all of the deliverables named above.

External Advisory Committee (EAC): A panel of four to six senior scientists with relevant expertise who are not PD(s)/PI(s) of a project involved in the Telomere Research Network that will provide expert input to the Steering Committee about the design and conduct of the methods comparison study.

Network Governance of Cooperative Agreement: Close interaction with the NIH to accomplish program goals.

NIH Award TERMS AND CONDITIONS

Motivation for the Methods Comparison Study

The Concerns:

- Differences in the literature regarding telomere length changes.
- Conflicting studies especially with environmental and psychosocial/socioeconomic stress exposures.

Our goal:
Development of best practice recommendations for population-based TL research

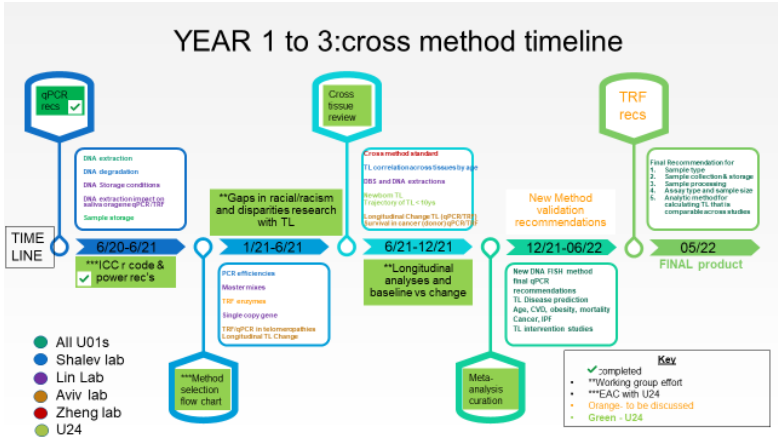
The Methods Comparison Studies (U01)

U01 Awarded Projects to serve three main functions:

1. Conduct a joint effort among telomere researchers to determine the relationship between different TL methods, inter-assay variability, and factors that influence findings.

2. Promote best practices for assays for TL measures for different types of studies, with a focus on population-based health research, including biological sample collection, storage, and processing, laboratory methods, data analysis, and reporting requirements.

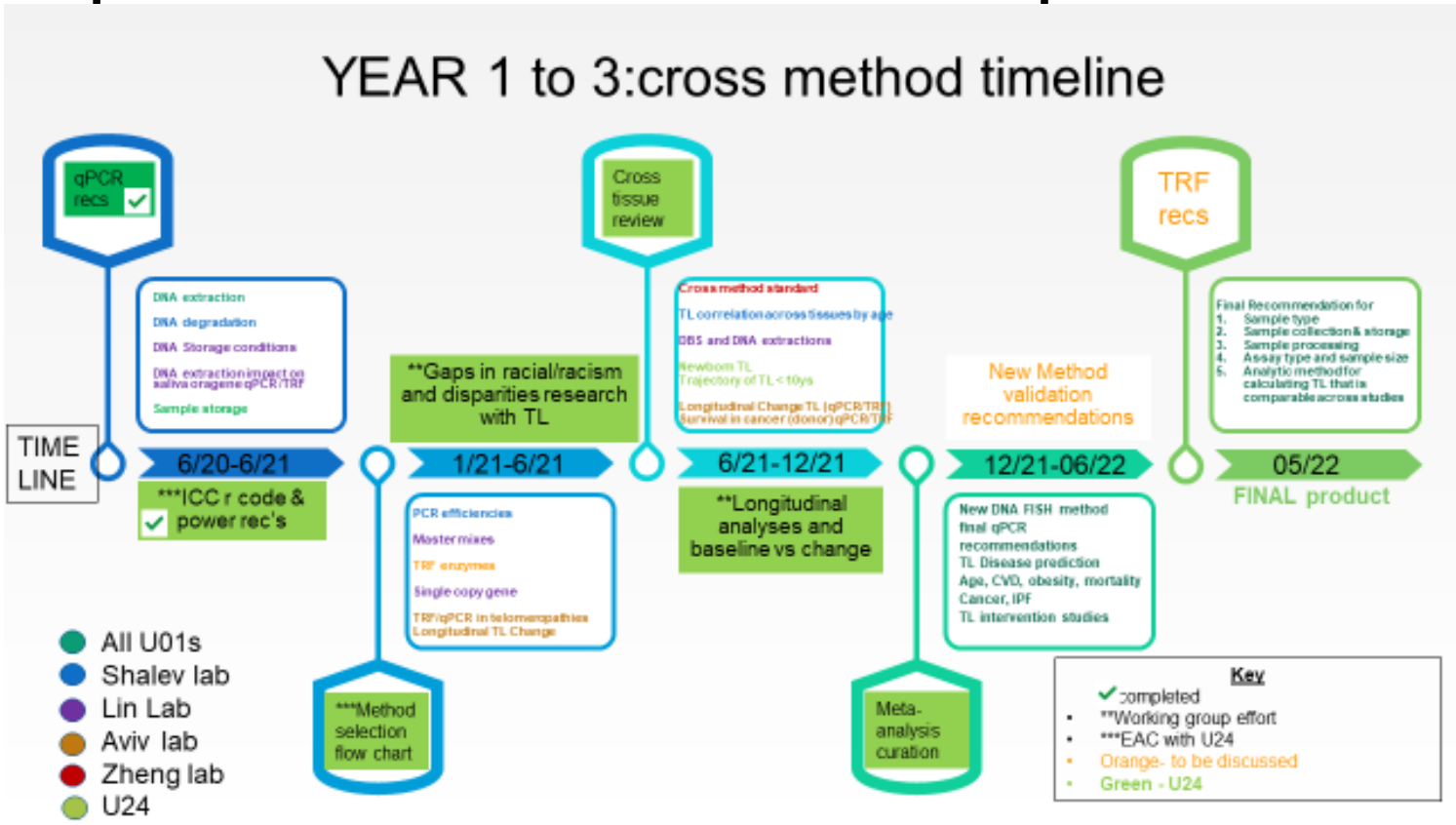
3. Finally, augment/repurpose existing methods or develop new methods to enhance the use of TL measurement.



The 4 U01 Projects awarded under this cooperative agreement will be <u>expected to conduct cross-validation to develop a set of recommendations for TL assays in terms of reproducibility and overall implication of findings depending on types of samples, storage methods, and sample work-up, and develop best practice recommendations for population-based TL research</u>. This will be done in coordination with the U24 Telomere Research Network/Collaboratory award

TELOMERE RESEARCH NETWORK

Components of Methods Comparison Study



Seeking feedback from External Advisory Committee at this meeting.

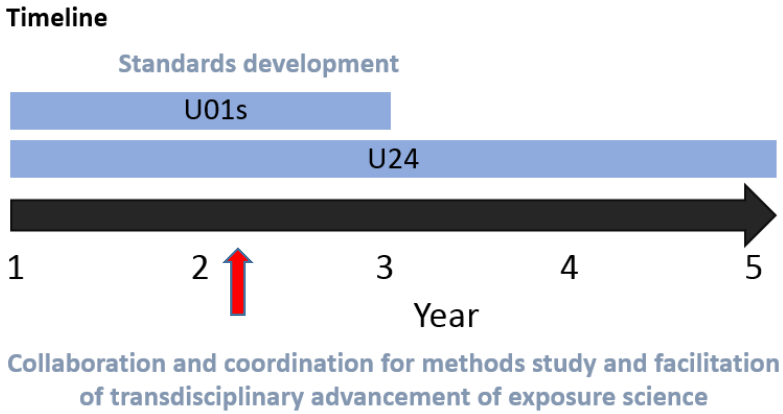
Use of NIH Samples to Address Key Questions

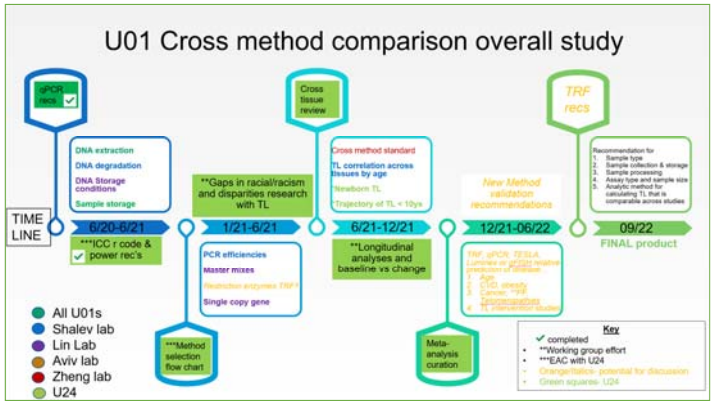
- Available blinded samples from NIA Intramural
  - 15 sets of test samples (consisting of ~35 subjects)
  - Age range 0-90
- How should we use it?
- When should we use it?

Seeking feedback from External Advisory Committee at this meeting.

TELOMERE RESEARCH NETWORK

Goals for this Meeting: Review and weigh in on our roadmap to meet our objective to develop a set of recommendations for TL measurement on a 3-year timeline





Project Period:
Sept 2019- May 2022

How to maximize our current efforts
What other aspects “need” to be addressed

The Telomere Research Network (U24)

RFA-AG-19-022: Research Network on Telomeres as Sentinels of Environmental Exposures, Psychosocial Stress, and Disease Susceptibility (U24 Clinical Trial Not Allowed)

U24 awarded project to serve three main functions (5-year timeline):

- Coordinate telomere length (TL) methods comparison involving labs supported under U01 awards in response to FOA (RFA-AG-19-023) to address the need for cross-validation between protocols and samples for establishing best practices for population-based TL research.
- Serve as Hub for organizing activities of this coordinated program, including development and dissemination of best practices based on Network activities, including developing recommended standards for the field for publishing and grant writing.
- Develop and foster an extended interdisciplinary Telomere Research Network (TRN) connecting the broader field through a flexible range of activities that will advance an interdisciplinary research agenda on telomeres and activities directly associated with TL maintenance as sentinels of environmental exposures, psychosocial stress, and disease susceptibility.

Goals for this Meeting: Review and Highlight TRN Activities

- Support the U01s' collaborative work
- Share resources and best practices with the field
- Showcase emerging research & chart new directions
- Support Pilot Projects
- Foster transdisciplinary dialogue

Seeking feedback from External Advisory Committee on activities related to methods comparison study.

TELOMERE RESEARCH NETWORK

Thanks from the NIH Team!

Max Guo, NIA

Michelle Heacock, NIEHS

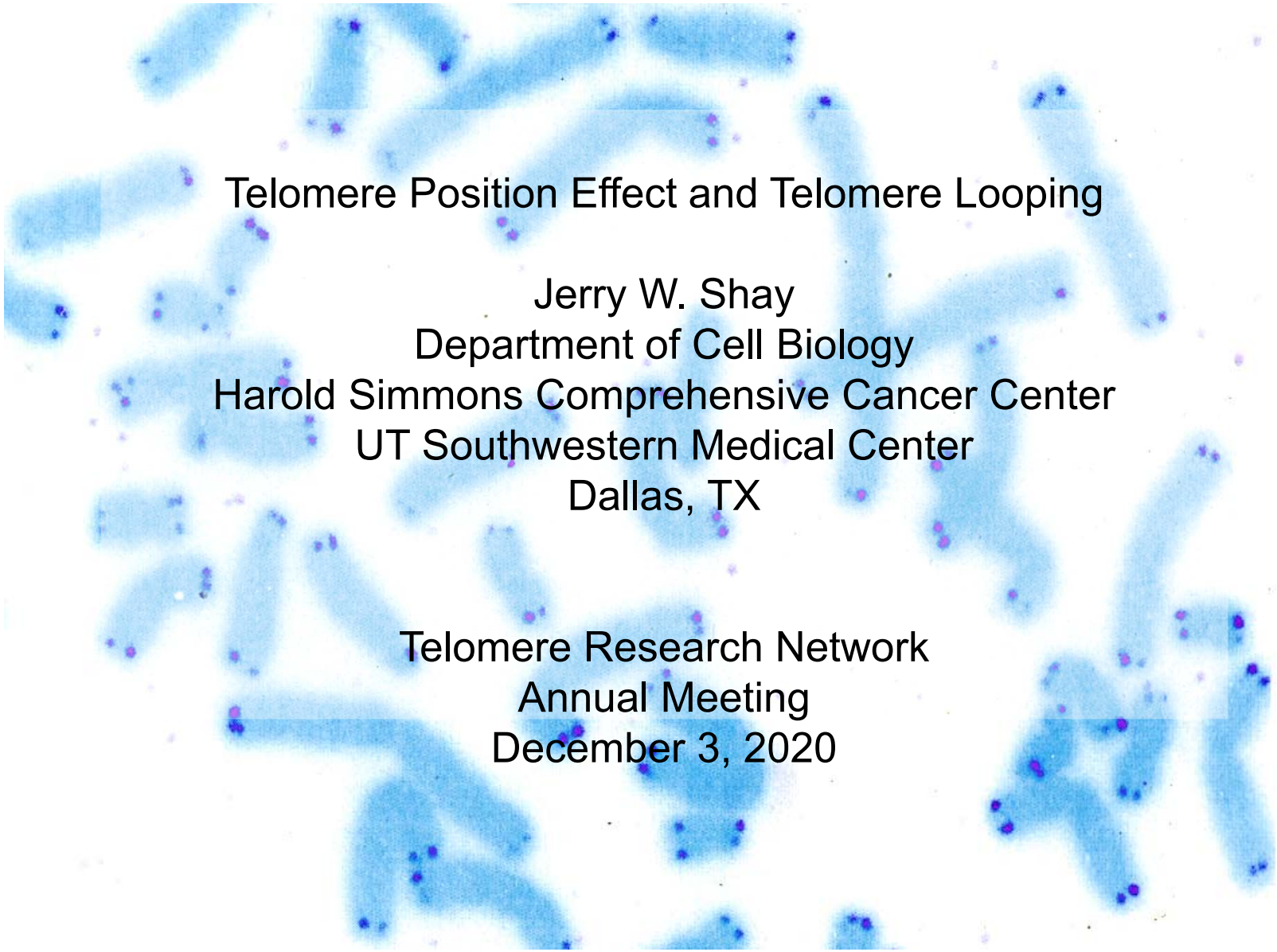
Lis Nielsen, NIA

Janine Simmons, NIA

Beyond telomere length: telomere position effects and telomere looping

Jerry Shay

TELOMERE RESEARCH NETWORK



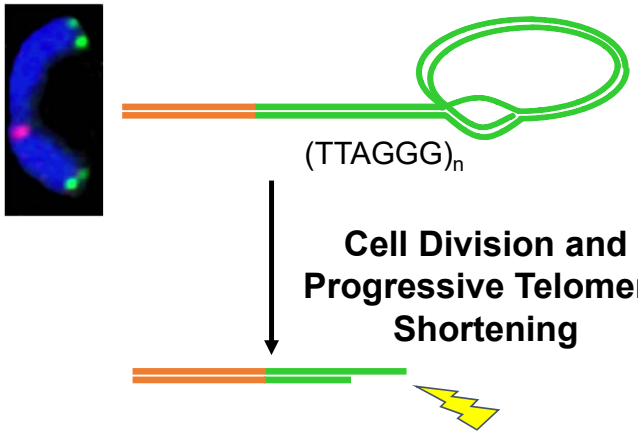
Telomere Position Effect and Telomere Looping

Jerry W. Shay
Department of Cell Biology
Harold Simmons Comprehensive Cancer Center
UT Southwestern Medical Center
Dallas, TX

Telomere Research Network
Annual Meeting
December 3, 2020

How Telomere Length Can Regulate Aging

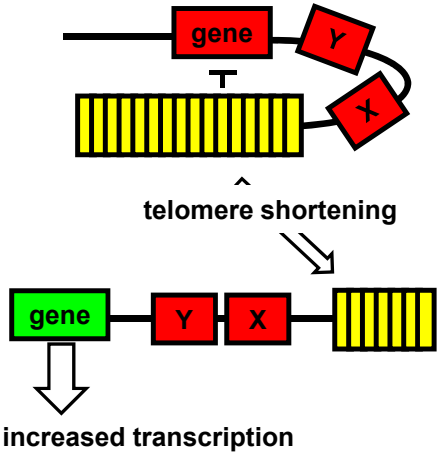
Telomere Uncapping and DNA Damage



DNA damage signal from a "too short" telomere

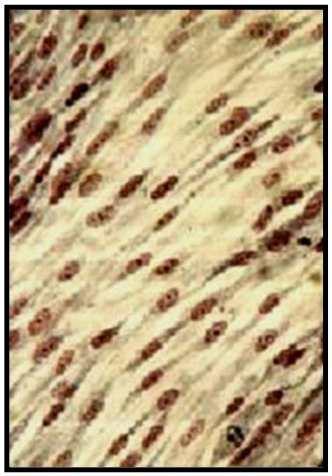
Telomere Looping Over Long Distances

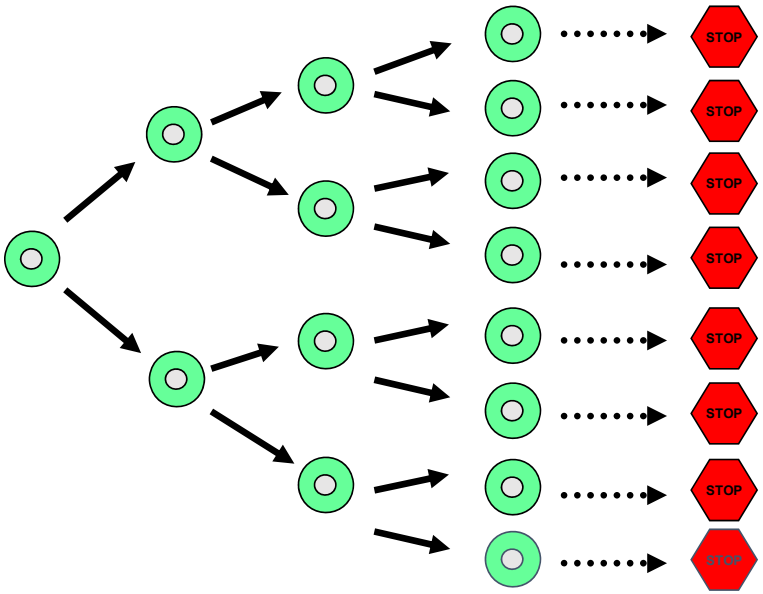
telomere length dependent looping: genes "far" from a telomere (TPE-OLD)



In Vitro: Replicative Senescence in Normal Cells

Hayflick, 1950s





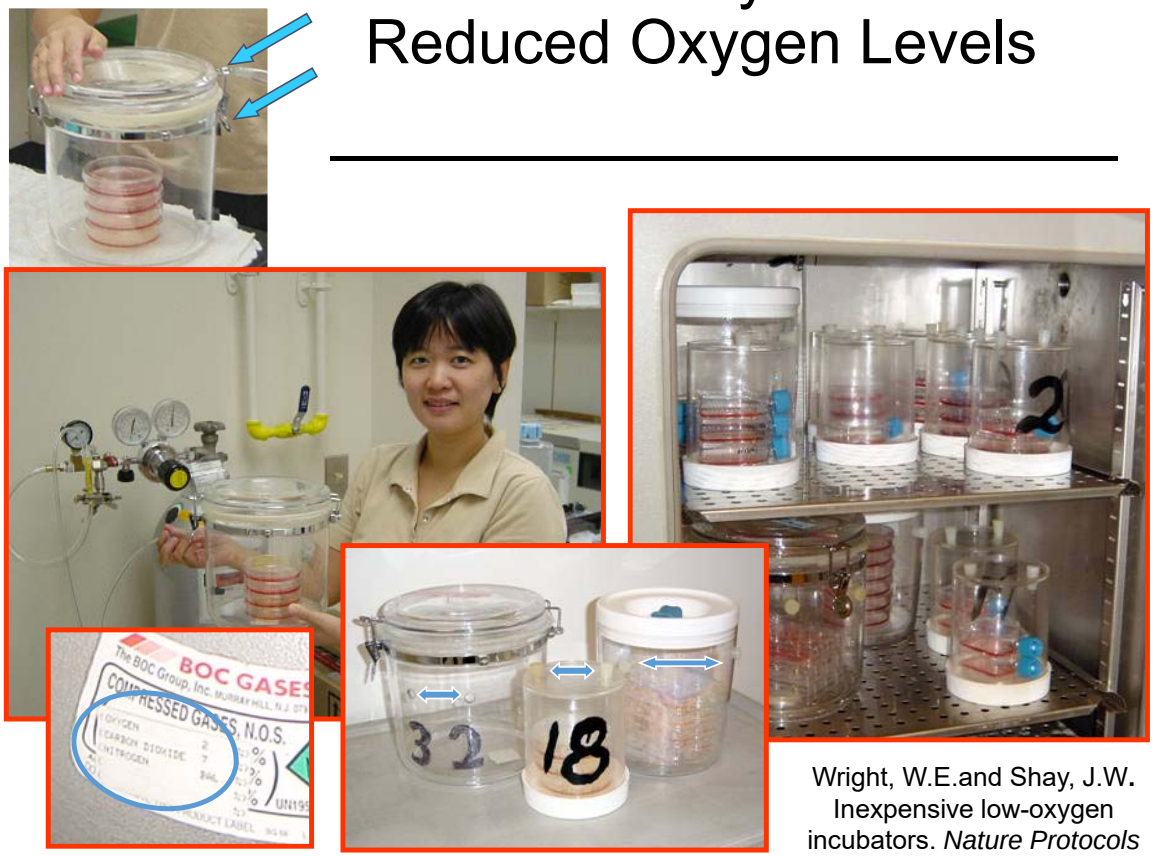
Short telomeres <u>correlate</u> with replicative senescence

Harley et al, *<u>Nature</u>* 345:458-460, 1990

Potential Problems of Telomere Measurements in Typical Culture Conditions

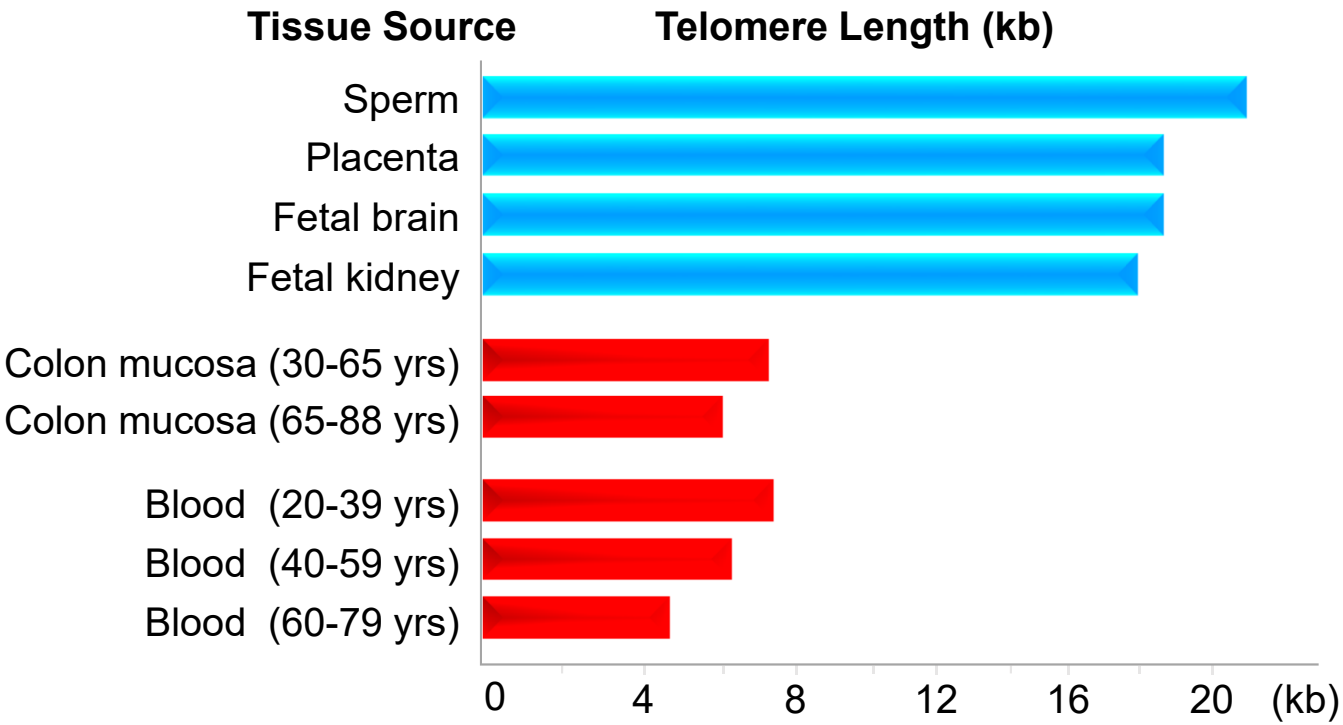
- Growing cells on plastic dishes in monolayer does not occur *in vivo.* Using extracellular matrices or irradiated fibroblasts increases cell lifespan.
- Growing cells in 21% oxygen is non physiological and culturing WI38 cells in 3% oxygen extends lifespan
- Growing cells in 10% serum is not physiological.
- Q: Why do telomeres lose 50bp per doubling *in vitro* but only 50bp per year *in vivo*? A: Culture shock

A Low Tech Way to Maintain Reduced Oxygen Levels



Wright, W.E.and Shay, J.W. Inexpensive low-oxygen incubators. *Nature Protocols* 1:4, 2008

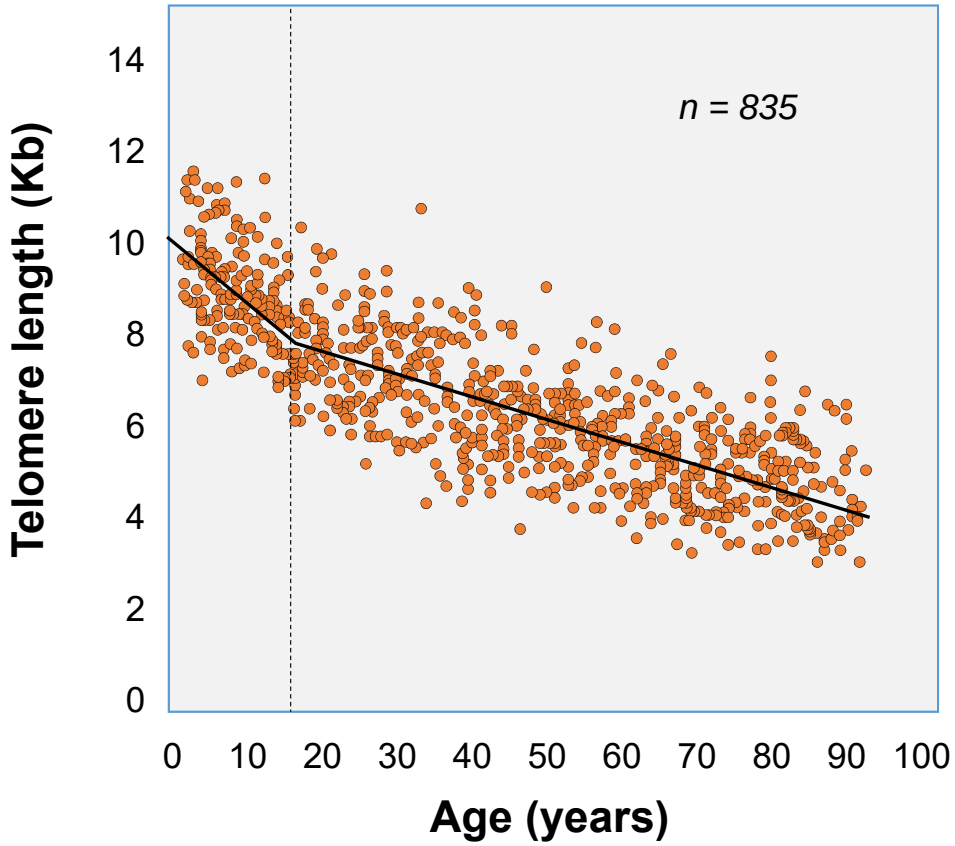
Progressive Telomere Shortening in Human Tissues



Short telomeres correlate with increased age

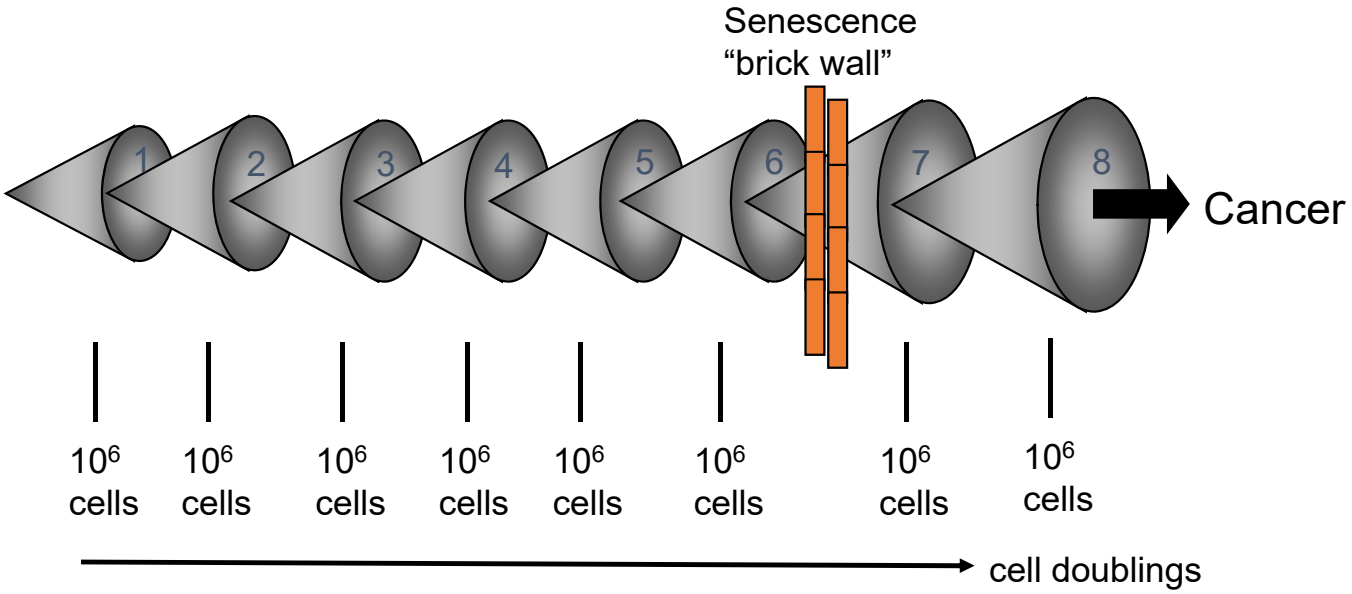
N. Hasti, R. Allshire, *Nature*, 346:866, 1990

Human Lymphocytes Show Progressive Telomere Shortening and Correlates with Increased Age



Aubert et al., *Plos Genetics*, 2012

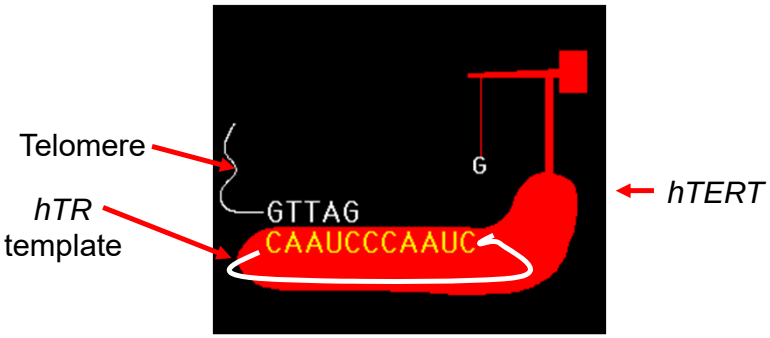
Replicative Senescence May Have Evolved as a Potent Anti-Cancer Protection Mechanism in Large Long-lived Species



Adverse Consequences of Short Telomeres

- Failure of stem cells to divide in sufficient numbers and loss of tissue renewal capacity.
- Senescence-associated secretory phenotype (SASP)
  - Increased inflammation
  - Decreased immune responses
- Increased risk of cancer (genomic instability) and other age-related diseases. Cancer cells that are immortal need to engage a telomere maintenance mechanism.

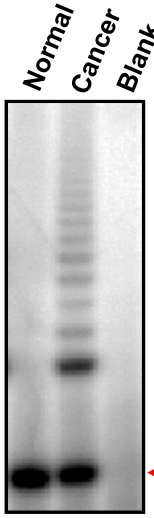
Telomerase is Detected in ~90% Human Cancers



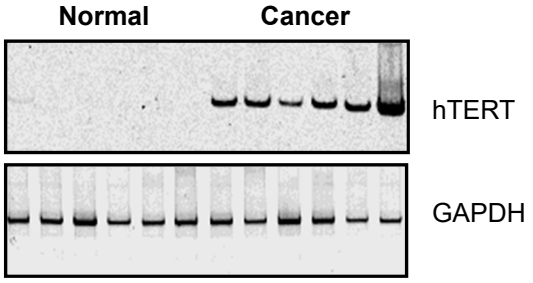
hTERT = human telomerase reverse transcriptase
(expressed in cancer cells and some normal stem cells)

hTR/hTERC = human telomerase template RNA
(constituitively expressed but up regulated in cancer)

Kim, N et al, Science 266: 2011-2015, 1994



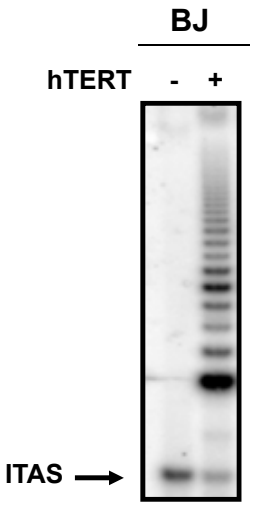
TRAP



Telomerase is a molecular motor that adds new DNA onto the ends of telomeres

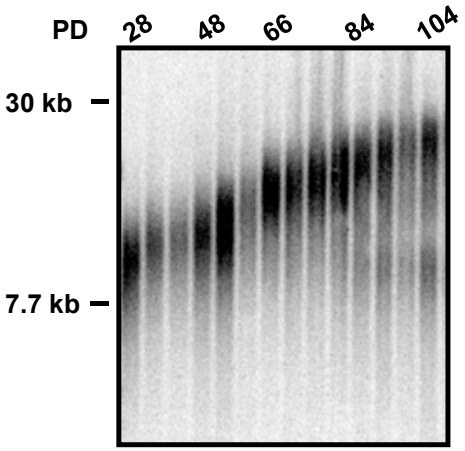
Telomerase Extends Cellular Lifespan: Immortalized Cells are Stable Long-term

Expression of hTERT in mortal cells is sufficient to generate telomerase activity, lengthen telomeres, and extend cellular lifespan



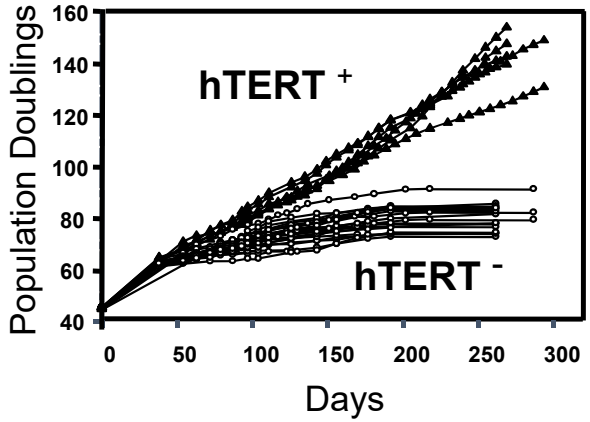
TRAP

Telomeric repeat amplification protocol



TRF

Terminal restriction fragments



ITAS = internal telomerase amplification standard
PD = population doubling

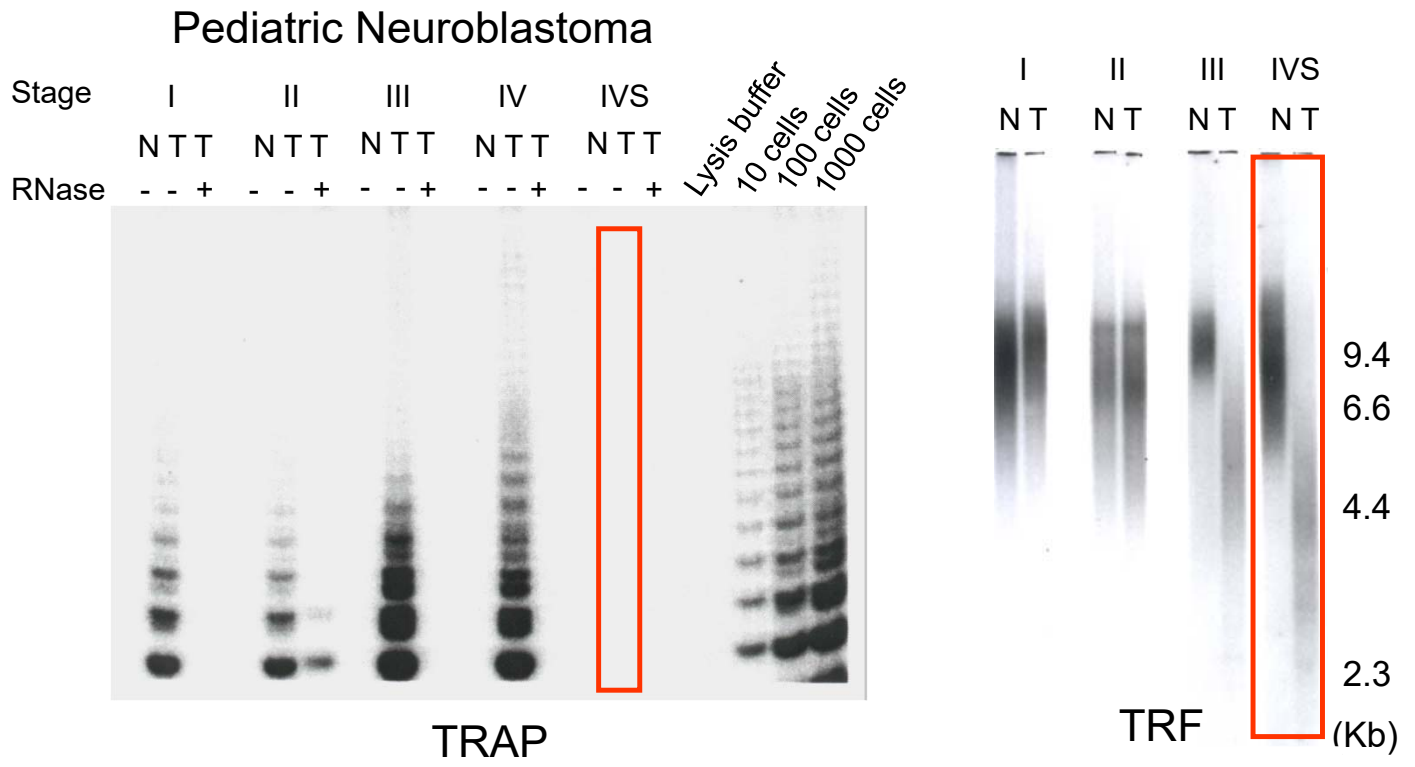
Bodnar et al, Science, 279:349 (1998)

Immortalization of Normal Cells with hTERT Does Not by Itself Transform Cells

Characteristics	***Normal***	***Cancer***	***hTERT*** $^{+}$
Contact inhibition of growth	present	absent	present
Growth factor requirements	high	low	high
Anchorage dependence	present	absent	present
Cell cycle checkpoints	intact	absent	intact
Karyotypic profile	normal	abnormal	normal
Tumors in nude mice	absent	present	absent
Proliferative life span	finite	indefinite	indefinite

Morales et al, Nature Gen 21:115, 1999

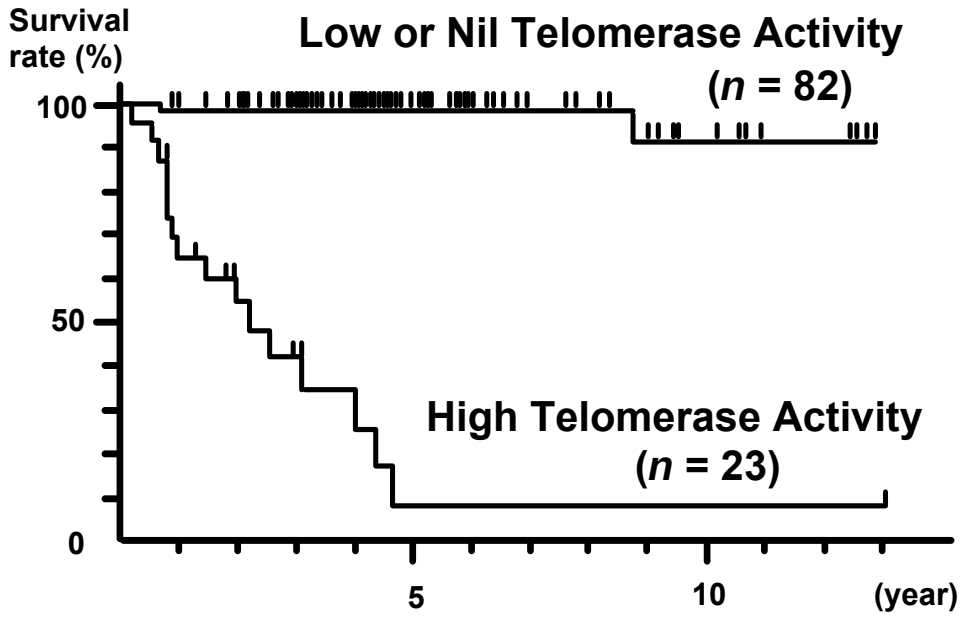
Some Malignant Tumors Do Not Have Telomerase Activity



What is the prognosis of patients with telomerase negative tumors?

Hiyama, E ….Shay, JW, *Nature Medicine 1: 249-257*

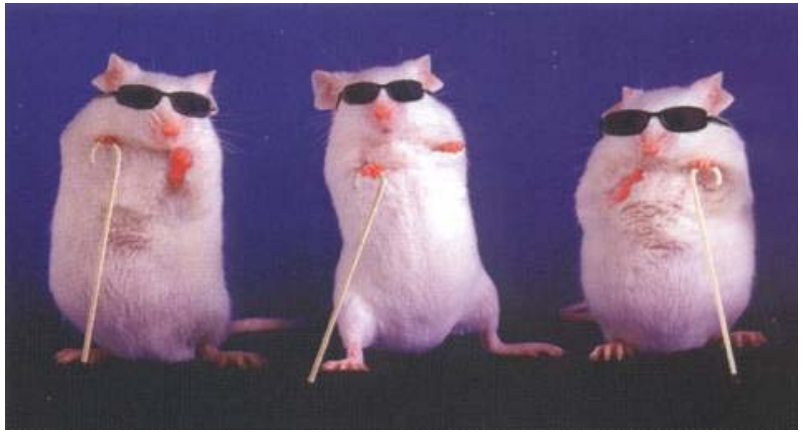
Individuals With Malignant Tumors Without Telomerase Activity Have Better Outcomes



Suggests inhibition of telomerase may be a potent anti-cancer approach

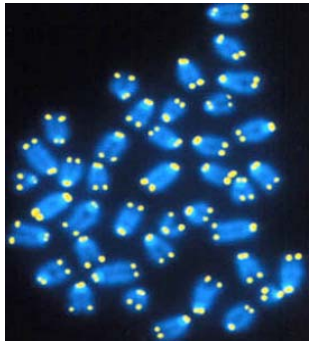
Hiyama et al, *Eur. J. Cancer*, 33:1932-1936.

Three (Telomerically Unchallenged) Blind Mice

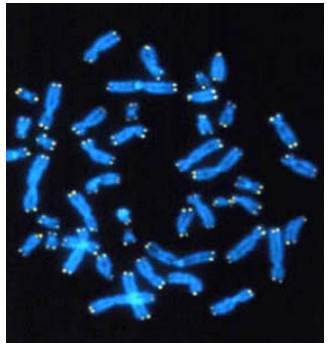


Inbred strains of mice have telomeres that are much longer than human telomeres

mouse



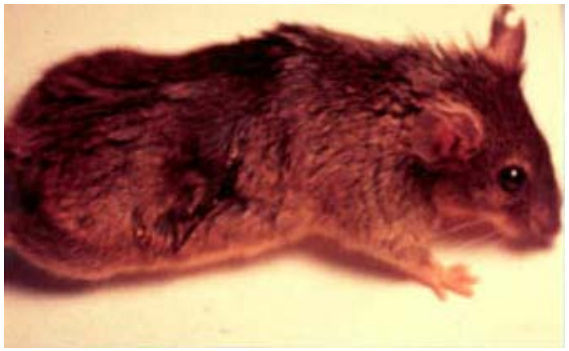
human



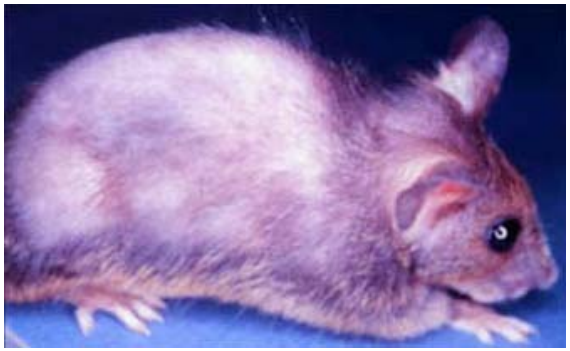
Of Mice and Men

- Normal mouse cells spontaneously immortalize with high frequency.
- Normal human cells do not spontaneously immortalize.
- Laboratory mice are 350 times smaller yet get cancer more frequently, per animal per year, than humans.
- Telomerase is repressed less effectively in mice.
- Humans may have cancer prevention mechanisms in effect during their reproductive life.

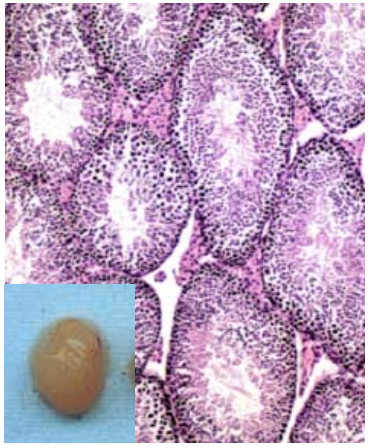
Aging Consequences of Telomere Dysfunction in Mice



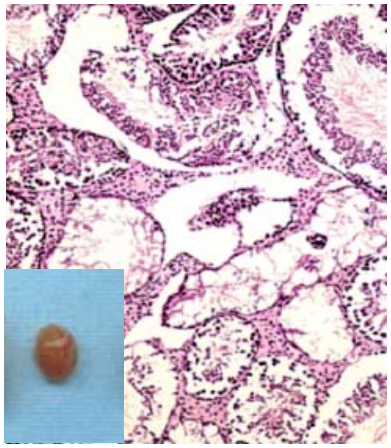
mTR+/+ 22 months



G3 mTR-/- 18 months



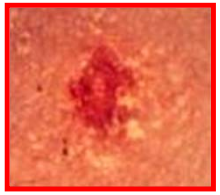
mTR +/+



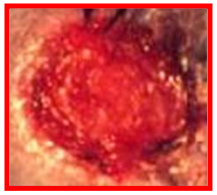
G6 mTR -/-

Wound Healing – Day 4

mTR +/+
(18 months)



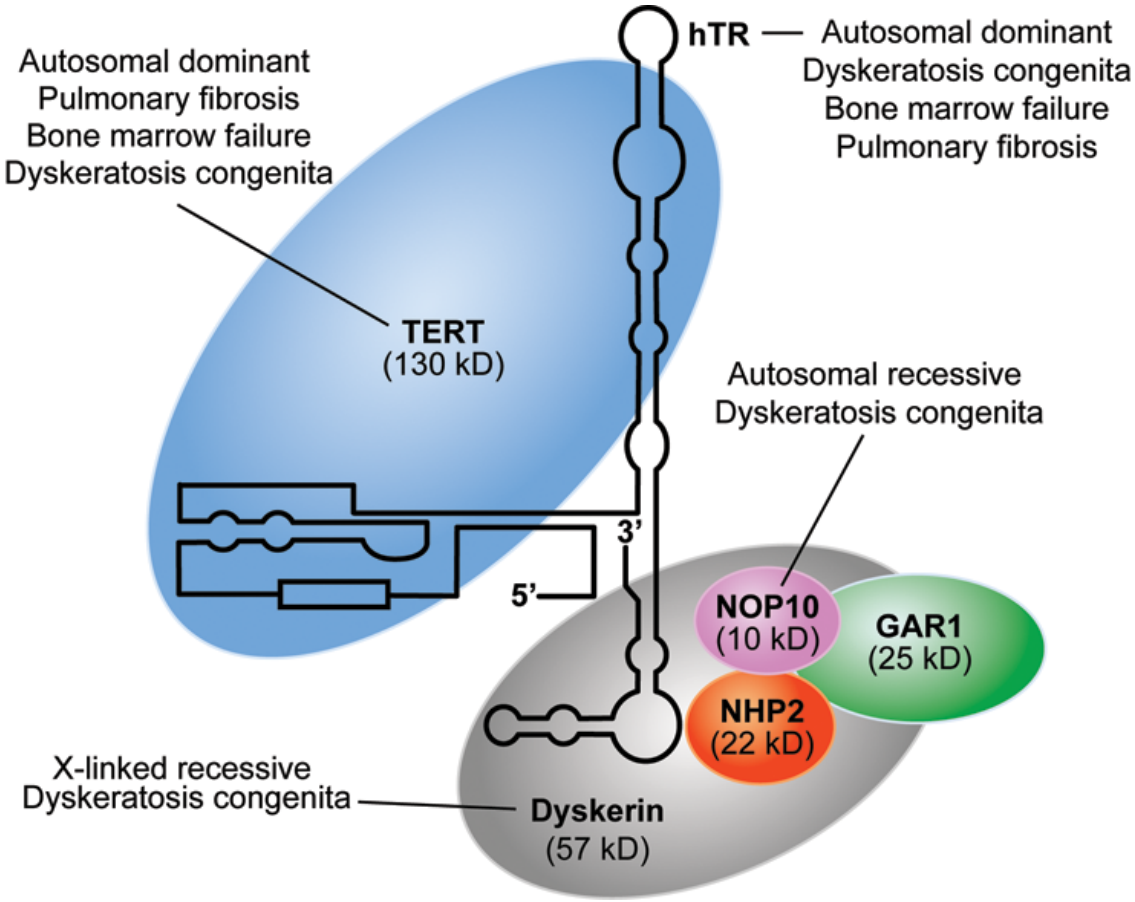
G6 mTR -/-
(18 months)



- Diminished mitogen lymphocyte proliferation
- Impaired hematopoietic reserve in bone marrow
- Increased apoptosis in GI tract

DePinho lab (Sandy Chang and Steve Artandi)

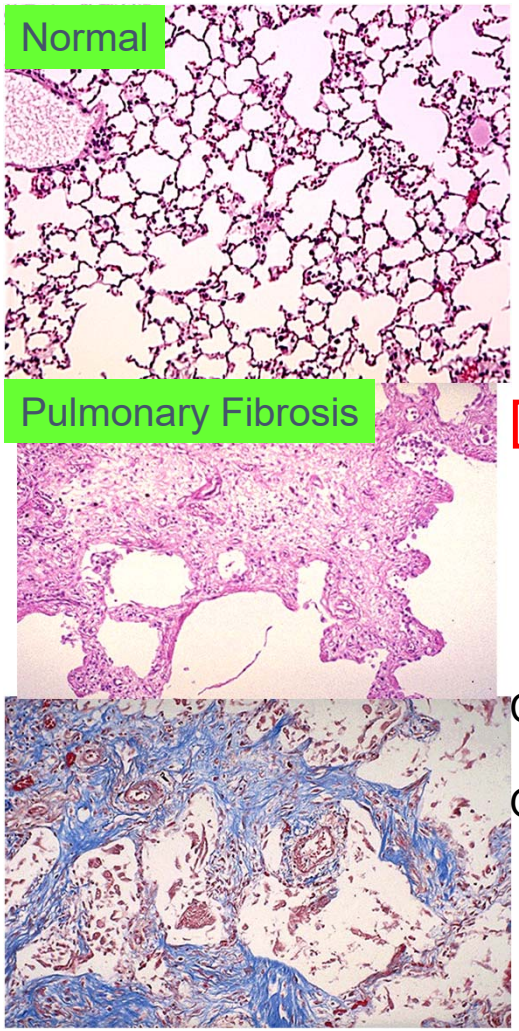
Mutations in Telomerase are Genetic Risk Factors for Clinical Disease: Telomeropathies



Dyskeratosis Congenita (DKC)

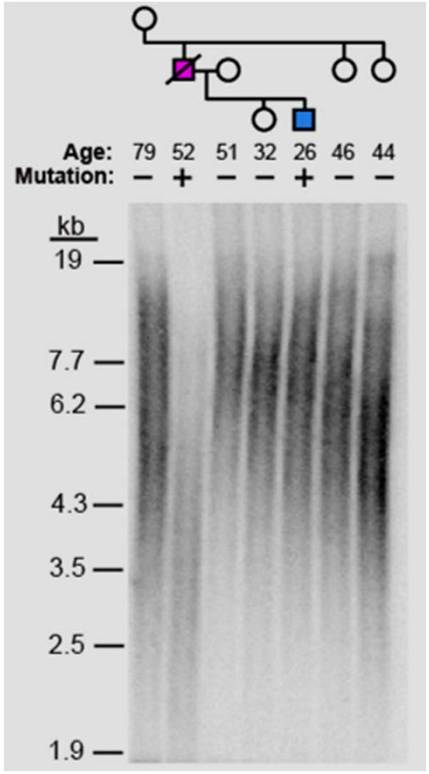
Organ system	Cells expressing telomerase	Clinical Defect
• Hair	proliferating hair follicle cells	alopecia
• Oral cavity	transient amplifying epithelium	leukoplakia
• Skin	basal epidermis (IFE)	nail dystrophy
• Liver	putative stem cells (oval)	cirrhosis
• Intestine	proliferating crypt cells	GI disorders
• Testes	spermatocytes	hypogonadism
• Bone marrow	proliferating stem cells	aplastic anemia

DKC is Also Associated with Pulmonary Disease

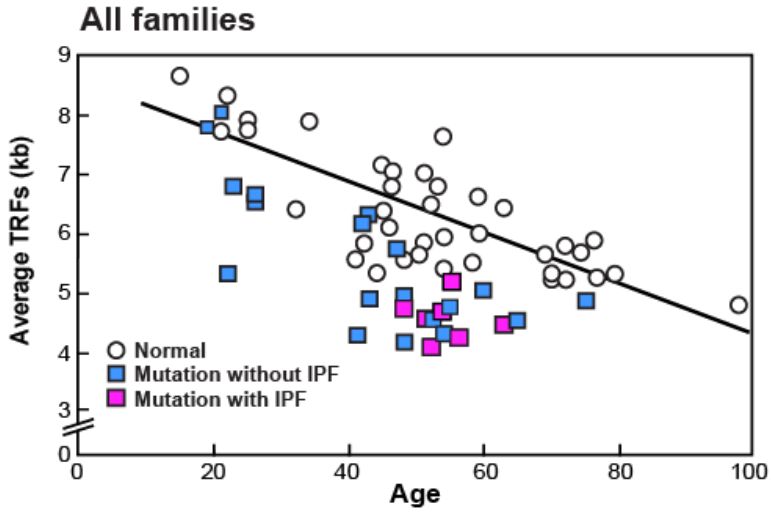


Abnormality	% Patients
Abnormal skin pigmentation	89
Nail dystrophy	88
Bone marrow failure	86
Leukoplakia	78
Excessive watering of the eyes	30
Learning difficulties	25
Pulmonary disease	20
Short stature	20
Extensive dental caries/loss	17
Esophageal stricture	17
Loss/gray hair/sparse eyelashes	16
Hyperhidrosis	8
Cancer	8
Liver disease/peptic ulceration	7
Osteoporosis	5

Peripheral Blood Mononuclear Cell Telomeres are Shorter in Patients With Idiopathic Pulmonary Fibrosis



Tsakiri et al. *PNAS, USA*, 104:7552-7557, 2007

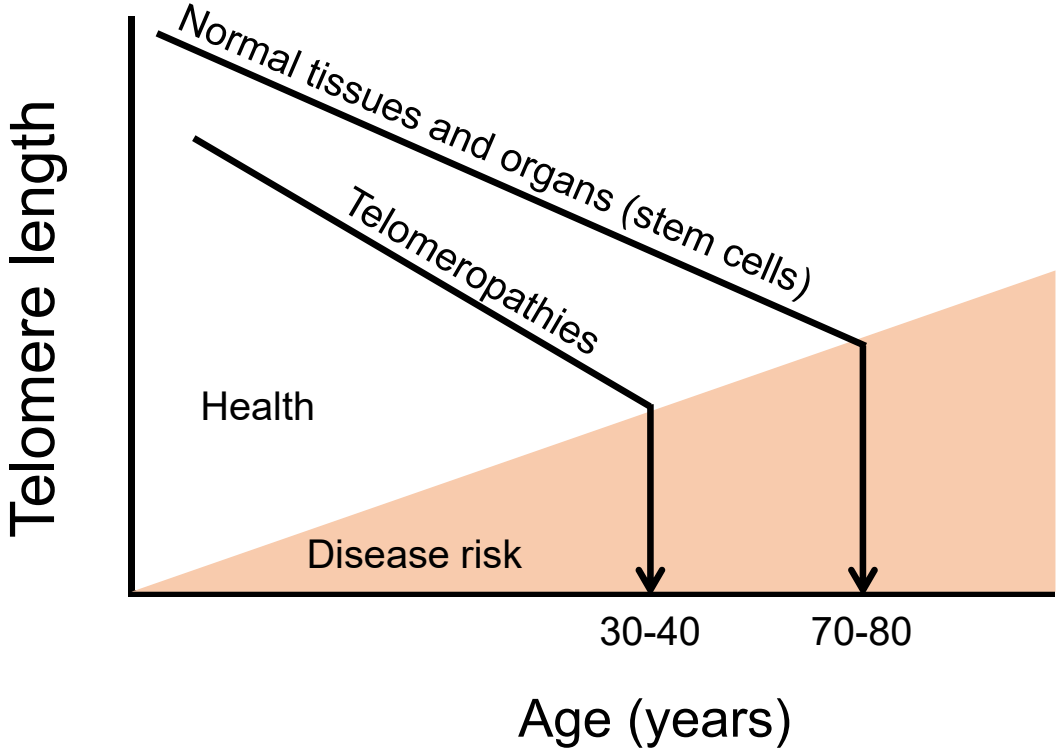


Normal

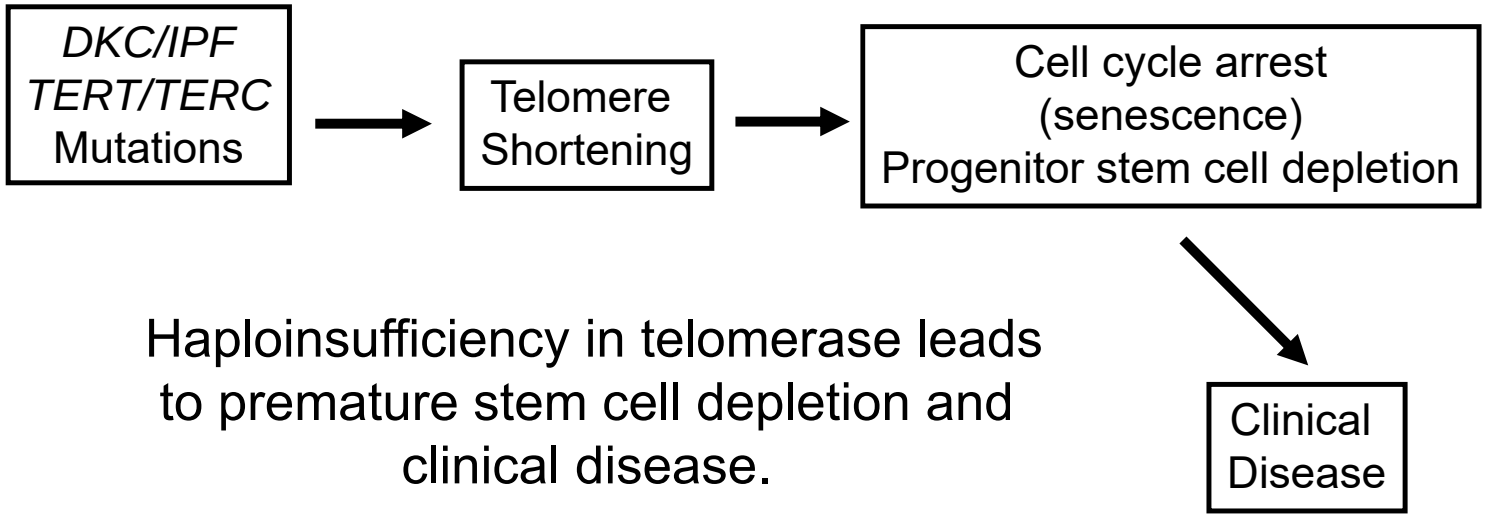
Mutation without IPF

Mutation with IPF

Telomere Length: Biomarker of Cellular Aging



Mutations in Components of the Telomerase Complex May Lead to Premature Stem Cell Depletion



Haploinsufficiency in telomerase leads to premature stem cell depletion and clinical disease.

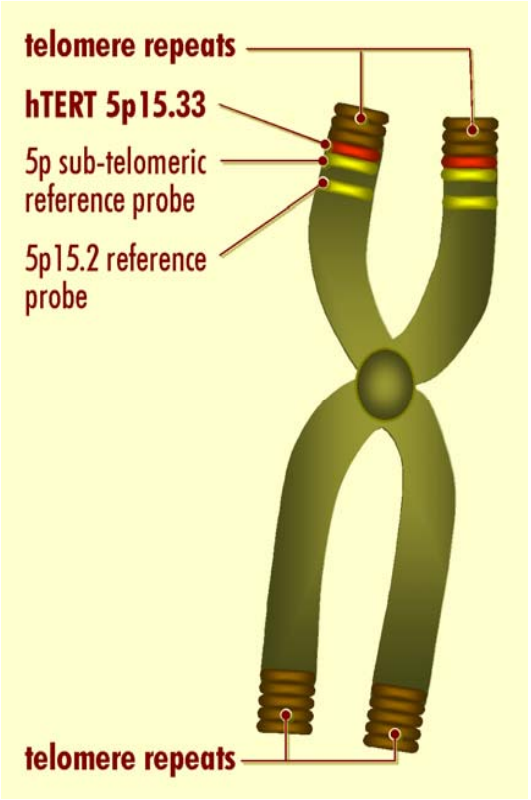
Telomerase is not in excess.

Mechanisms Involved in Telomerase Regulation

While telomerase activity is expressed during the first trimester in human development, it is poorly understood what causes its repression in normal somatic cells or its reactivation in cancer.

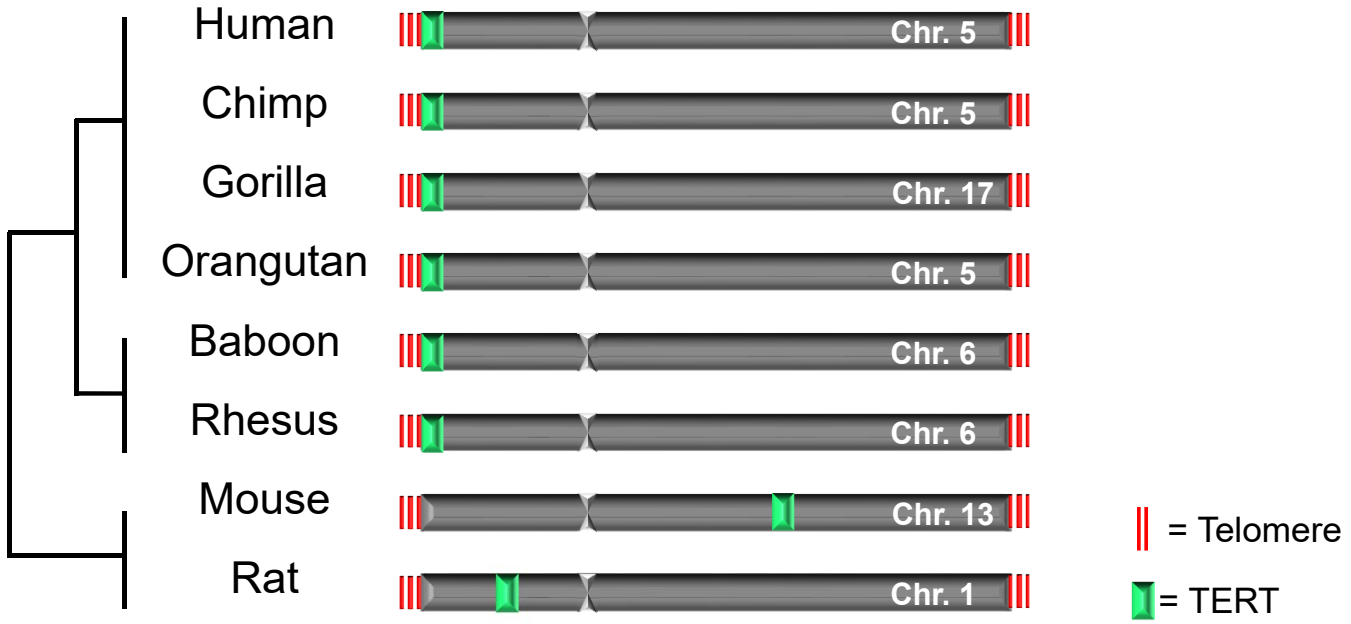
- Transcription factors?
- Alterations in TERT?
  - TERT promoter mutations
  - TERT rearrangements
  - TERT amplifications
- Alternative splicing?
- Epigenetic modifications?

Wong et al, *Cell Reports*, 2013
Wong et al, *Nature Comm*, 2014
Robin et al, *Genes and Dev*, 2014
Kim et al, *PLoS Biol*, 2016



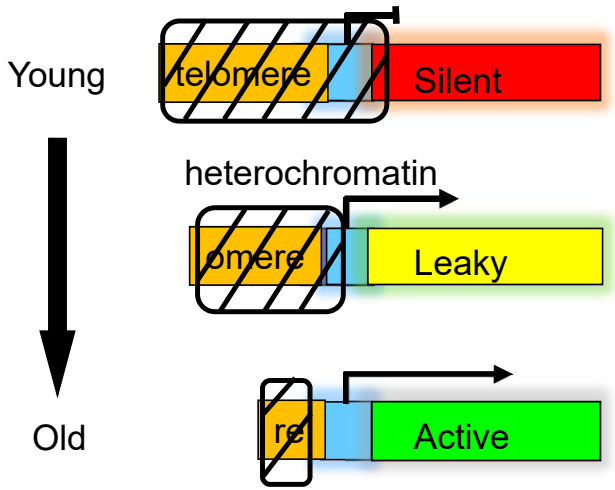
W. Nicol Keith et al, *Neoplasia*, 2000

Conserved Location of the *TERT* Gene in Higher Primates ~1Mb from the Telomere



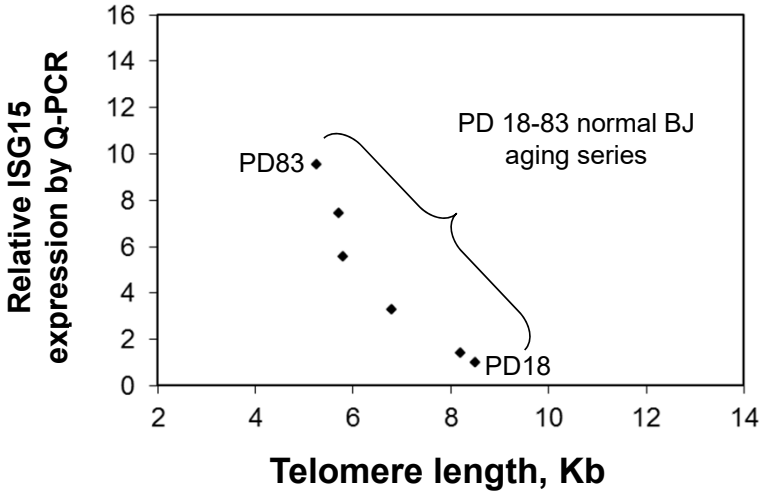
Does having a telomere adjacent to *hTERT* limit the maximal length of human telomeres and make telomerase more easily expressed during cancer progression when telomeres are short?

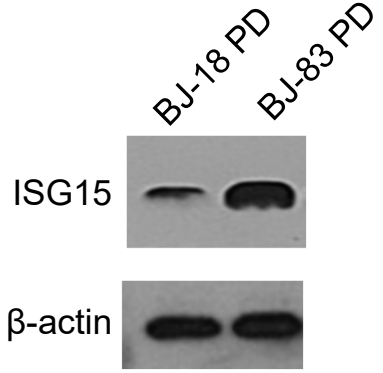
What Mechanisms are Involved in Short Telomere-induced Signaling?

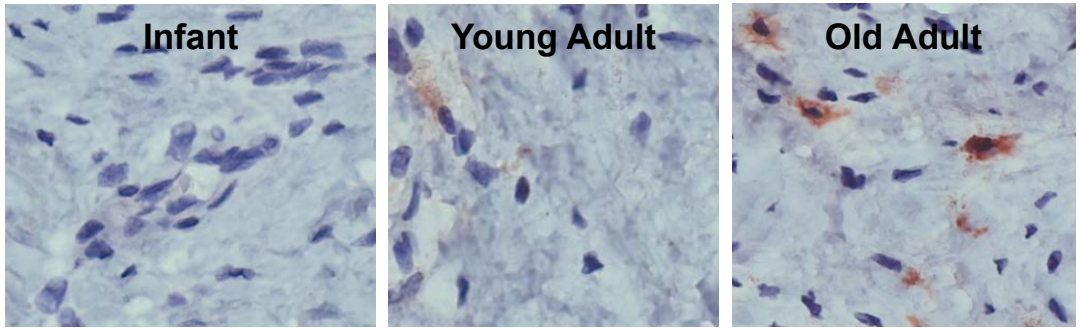


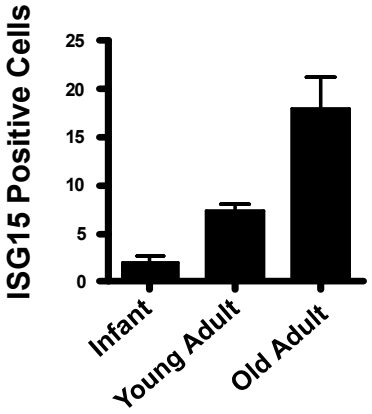
Telomere Position Effect (TPE)

Differential Expression of Young Versus Old Cell RNA Identified ISG15





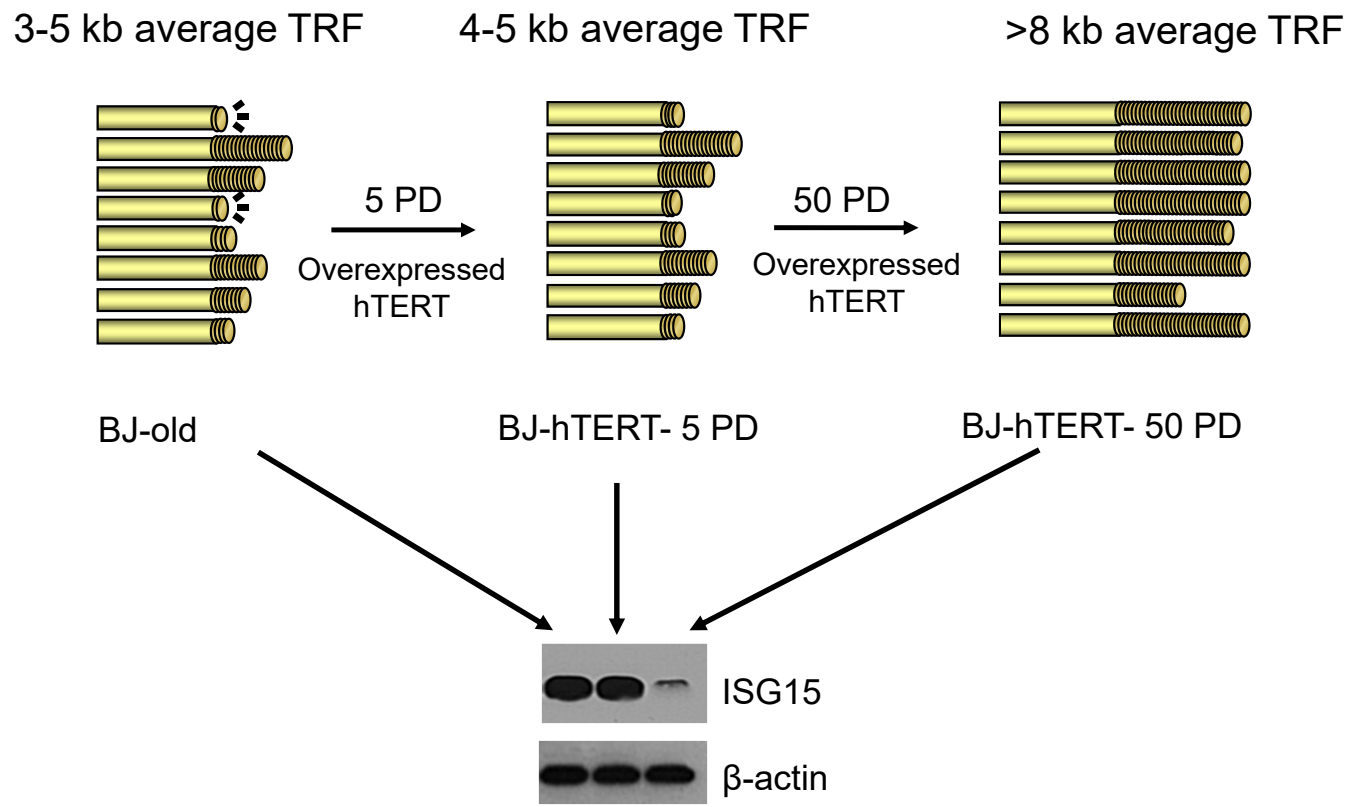




Lou et al., *Aging*, 1:608-621

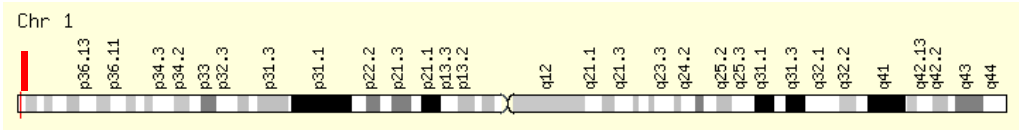
Human Skin (ISG15)

Elongation of Telomeres Inhibits the Expression of ISG15 in BJ Cells

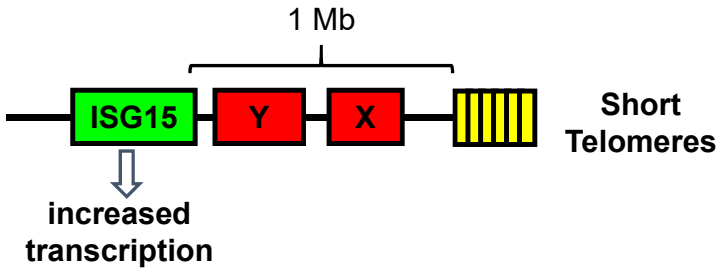


Is ISG15 (G1P2) Being Regulated by Classic TPE?

- Located on 1p36.33 (~1 Mb from the telomere) and functions as a ubiquitin-like protein believed to play a key role in the innate immune responses to viral infections.



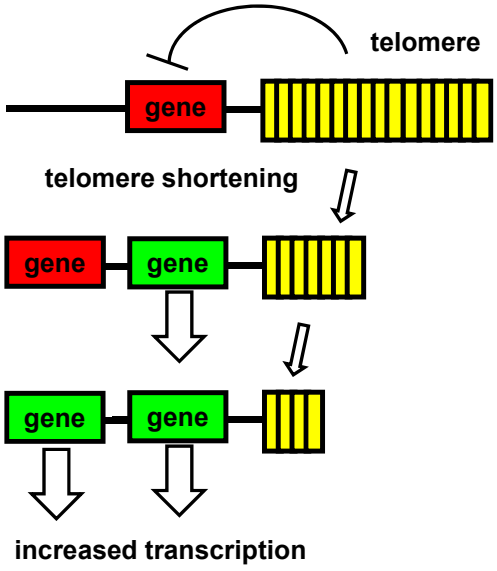
- Other functions: May be involved in RNA splicing, chromatin remodeling, and stress responses.
- We discovered that several genes tested between ISG15 and the telomere were not regulated by classic TPE



How Telomere Length Can Regulate Transcription

Classic TPE (telomere position effect)

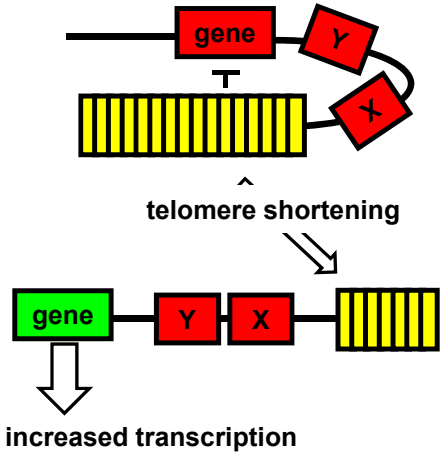
telomere length dependent position effect: genes "close" to a telomere



Baur et al, *Science*, 2001
Stadler et al, *Nature Struct and Mol Biology*, 2013

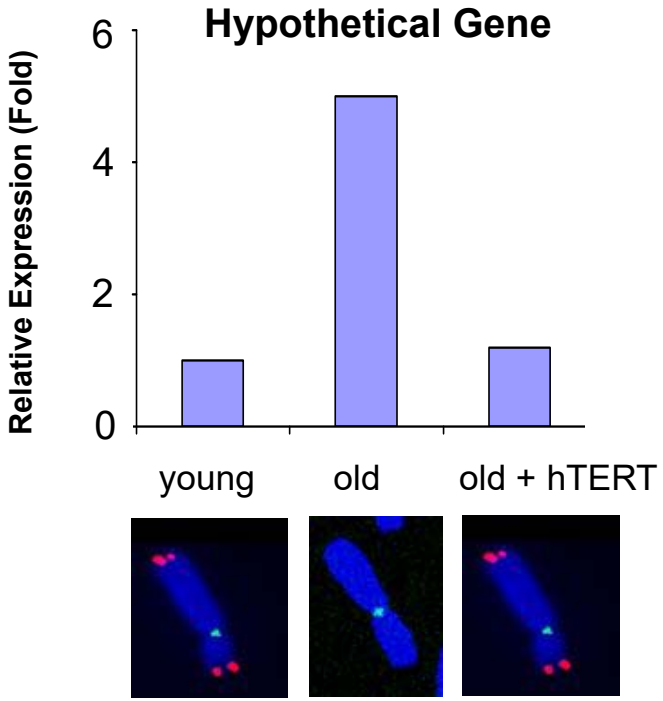
Telomere Looping Over Long Distances

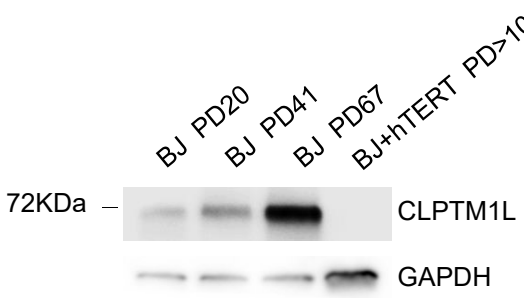
telomere length dependent looping: genes "far" from a telomere (TPE-OLD)



Robin et al, *Gene Dev.*, 2014
Robin et al, *Genome Research*, 2015
Kim etal, *PLoS Bio.*, 2016

Cleft Lip and Palate Transmembrane Protein 1-Like (CLPTM1L) is a TPE-OLD Regulated Gene

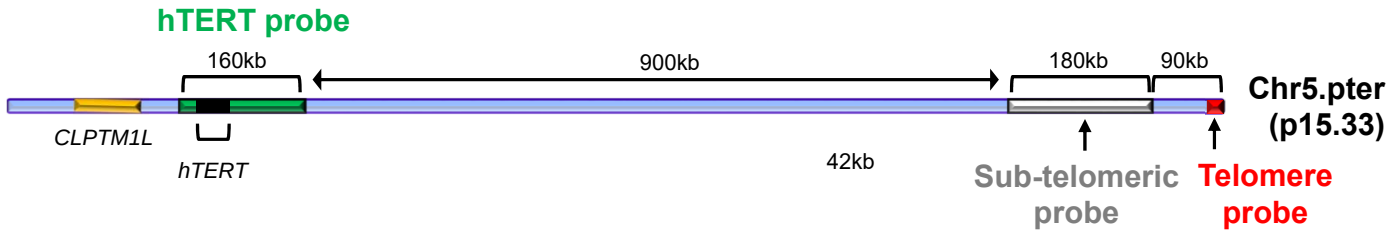


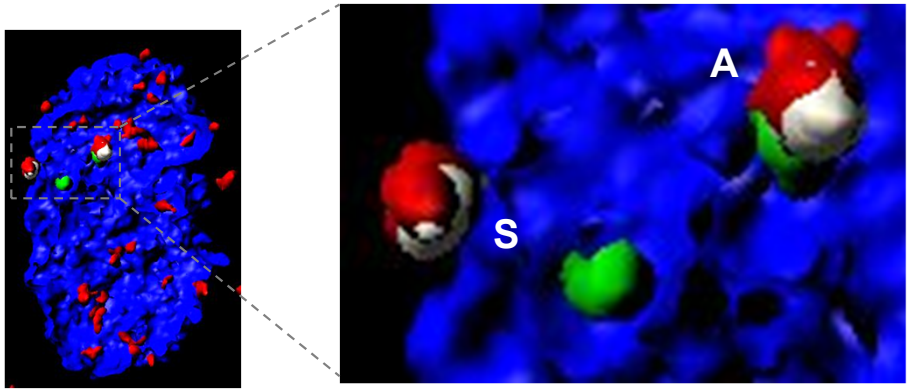


Wanil Kim

- CPP9p (cisplatin resistance-related protein 9).
- Highly expressed in 15 distinct cancers.
- Goes up in aging and cancer and stays up.
- Very close to the hTERT locus.

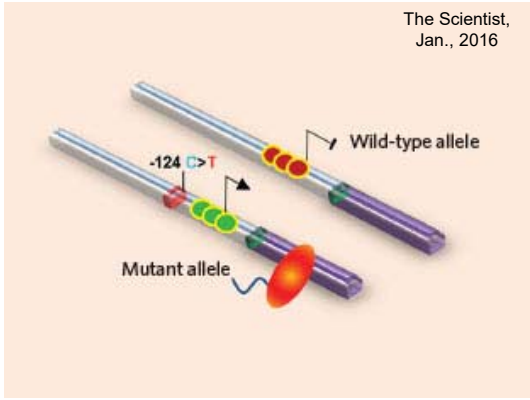
Three-dimensional Interactions Between the hTERT Locus and the 5p Telomere by Length





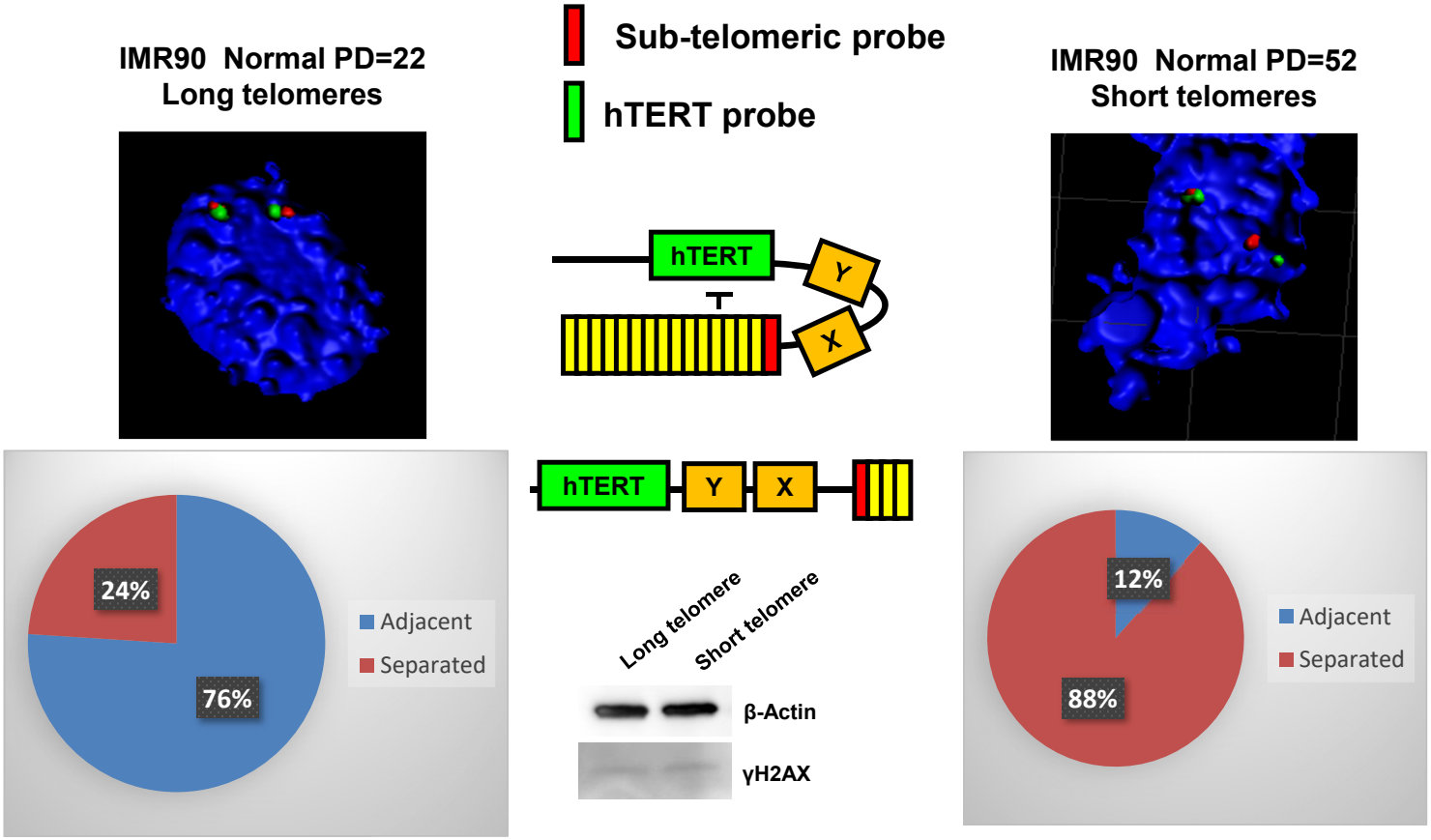
BJ Fibroblasts PD 60

Telomere: Red
Subtel-5p: Far-Red
hTERT: Green
DNA: Blue

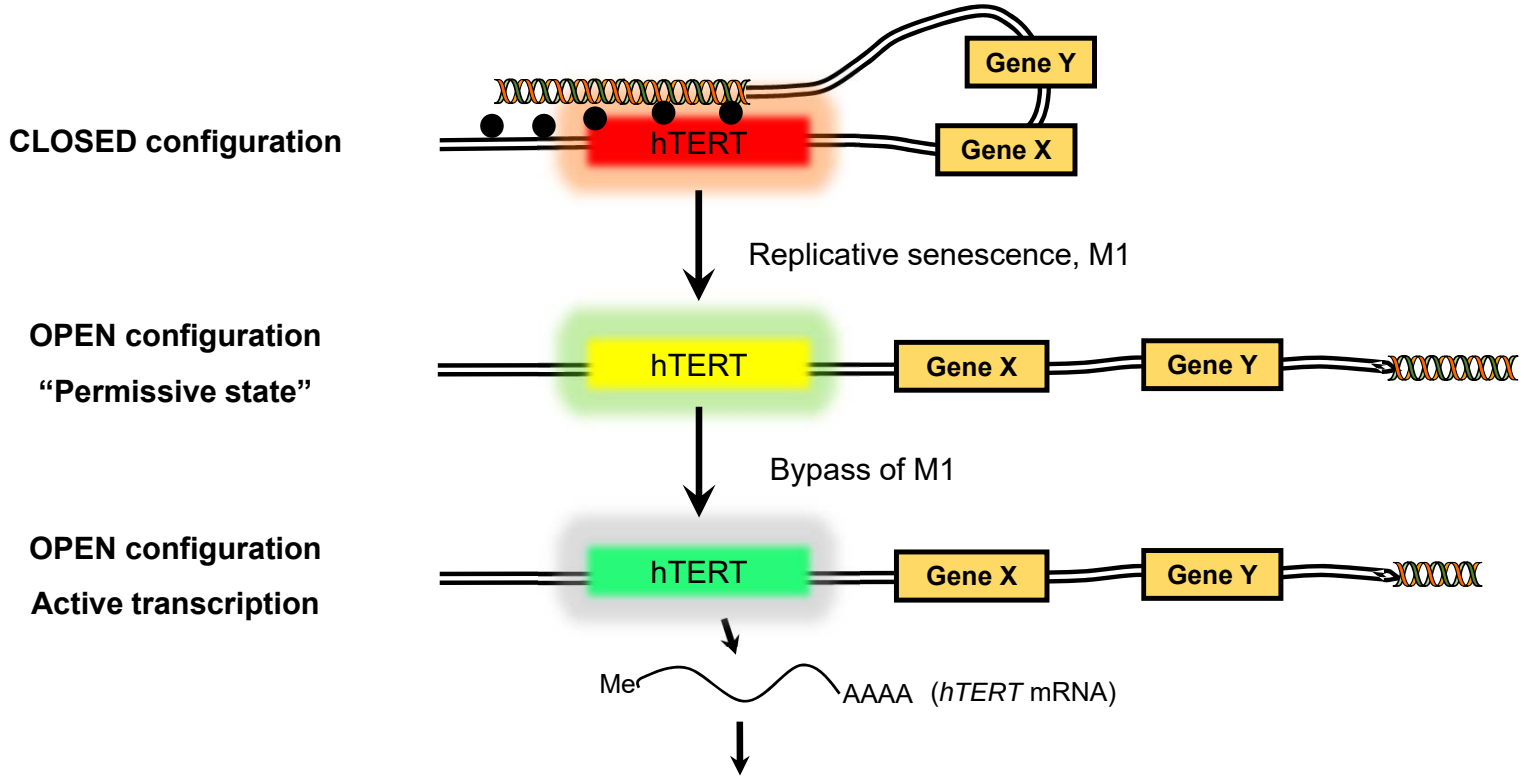


In cancer only one TERT allele is mutated

The Proximity of the Terminus of Chromosome 5p and hTERT Changes During *in Vitro* Aging



Working Model for Telomere Looping in Human Development and Aging



TERT promoter mutation or splicing change = telomerase enzyme activity

Conclusions

- Telomere looping (TPE-OLD) occurs in a number of human genes and provides a mechanism to regulate aging by progressive telomere shortening without the induction of a DNA damage signal.
- Telomere looping occurs between the human TERT locus and chromosome 5p terminus in cells with long telomeres.
- Telomere looping is disengaged with at least one TERT locus in normal cells with short telomeres.
- Loss of telomere looping correlates with under-methylation of the hTERT locus, more active histone marks, and TERT transcription.

PLoS Biology e2000016. doi: 10.1371/journal.pbio.2000016

Telomere Length as a Biomarker of Healthy Aging in Centenarians

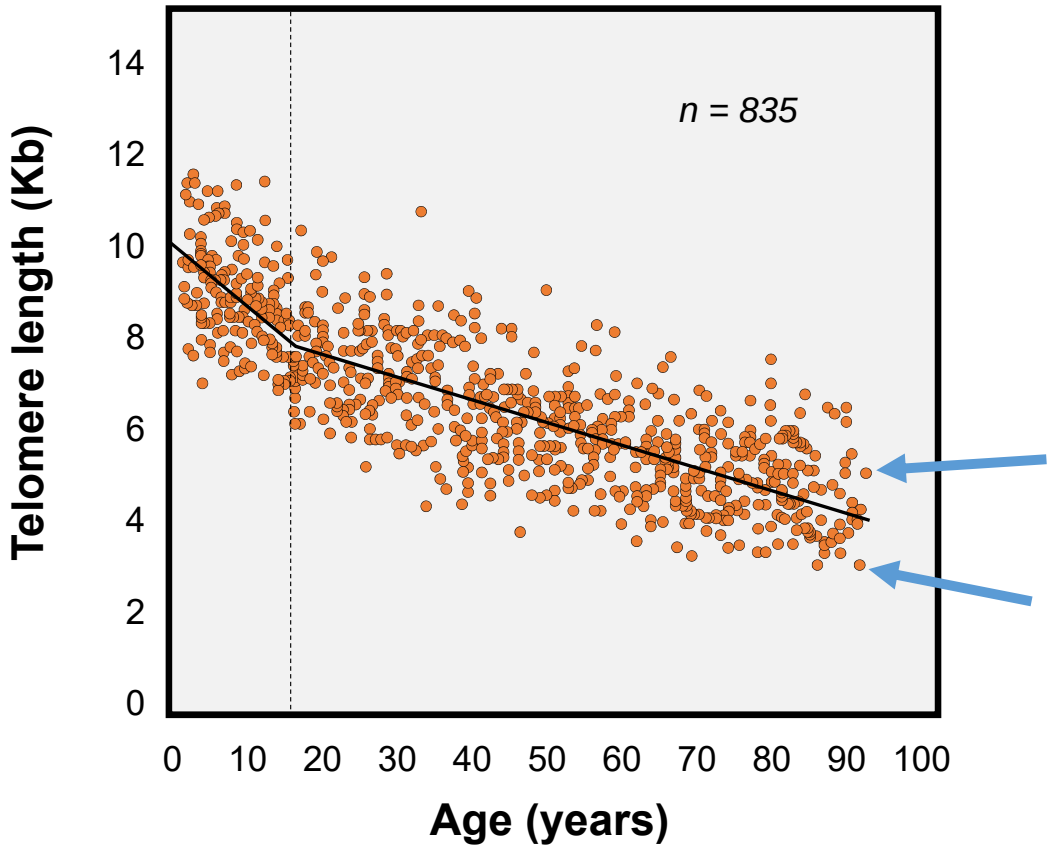
Enzo Tedone

Ejun Huang

Centenarians that Escape or Postpone Age-related Diseases May Permit the Identification of Biomarkers of Healthy Aging

- There are ~500,000 centenarians (100+ years old) in the world (30% chance to reach 90 and if so, ~1% will make 100).
- The probability to live healthily past the age of 100 years is very low, as most centenarians are affected by age-related diseases and often reside in long-term nursing facilities.
- Some exceptional high performing centenarians reach the extreme limits of human lifespan by escaping almost all major age-related diseases.
- Previous studies have generally pooled all centenarians and have focused on the study of resting/unstimulated peripheral blood mononuclear cells (PBMC).

Human Lymphocytes Show Progressive Telomere Shortening that Correlate with Increased Age

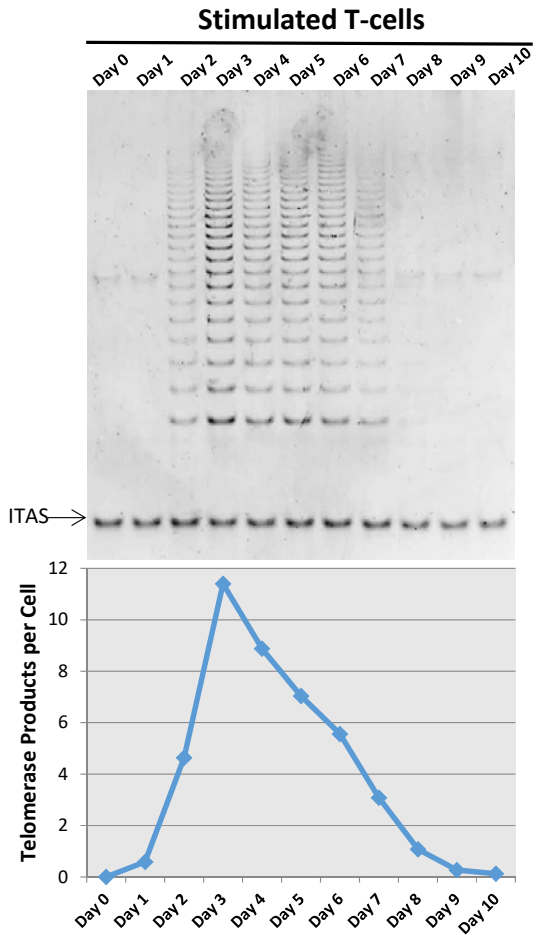


Aubert et al., *Plos Genetics*, 2012

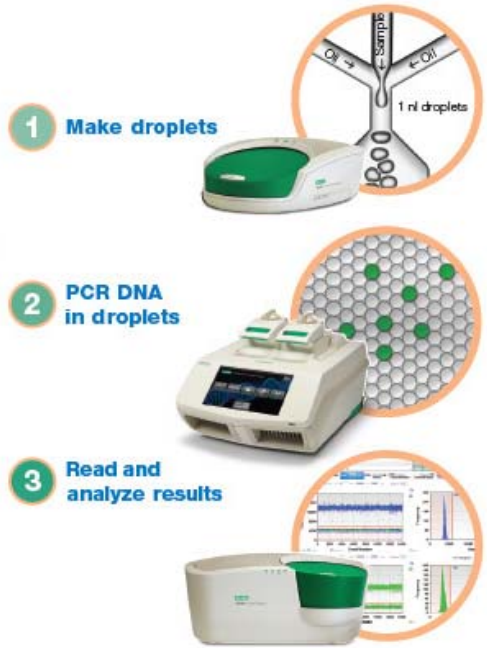
Combining TRAP Assay and Droplet Digital PCR to Measure Telomerase Activity Quantitatively

Gel-based TRAP

ddTRAP

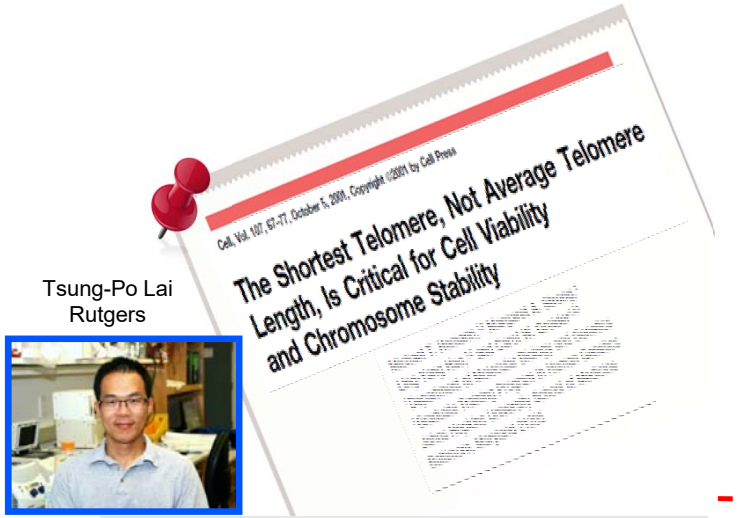


Droplet Digital PCR (ddPCR)



Andrew Ludlow, et al., Nucleic Acids Research, 2012

Polyps and Malignant Cancers Have More Short Telomeres Compared to Normal Tissues



Tsung-Po Lai
Rutgers

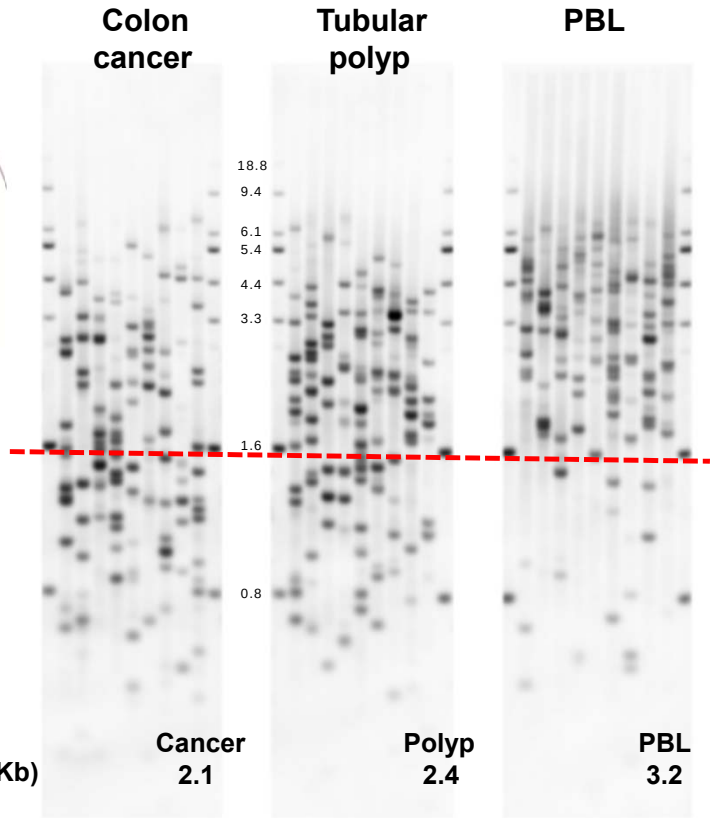
TeSLA:
Telomere Shortest Length Assay

ARTICLE

OPEN

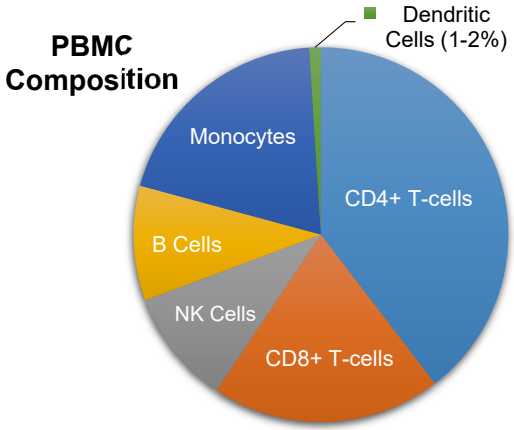
A method for measuring the distribution of the shortest telomeres in cells and tissues

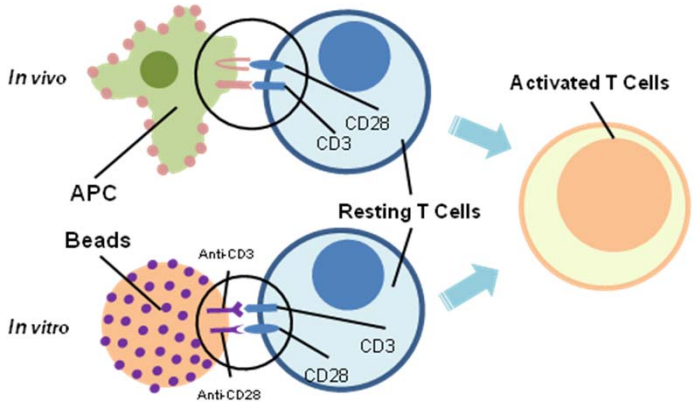
Tsung-Po Lai[1], Ning Zhang[1,2], Jungsik Noh[2], Ilgen Mender[1], Enzo Tedone[1], Ejun Huang[1], Woodring E. Wright[1], Gaudenz Danuser[1,2] & Jerry W. Shay[1]

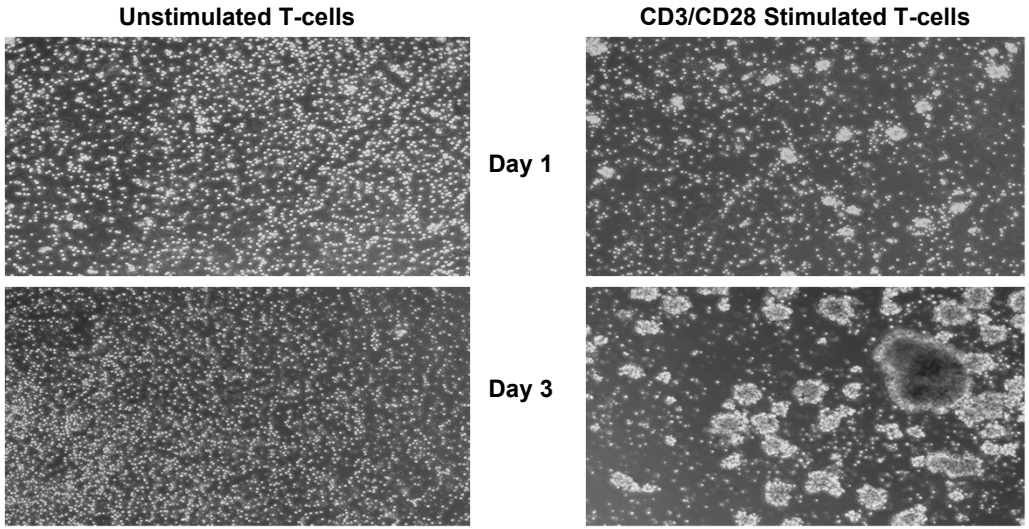


	Cancer	Polyp	PBL
Average (Kb)	2.1	2.4	3.2
< 1.6 Kb (%)	62	39	19

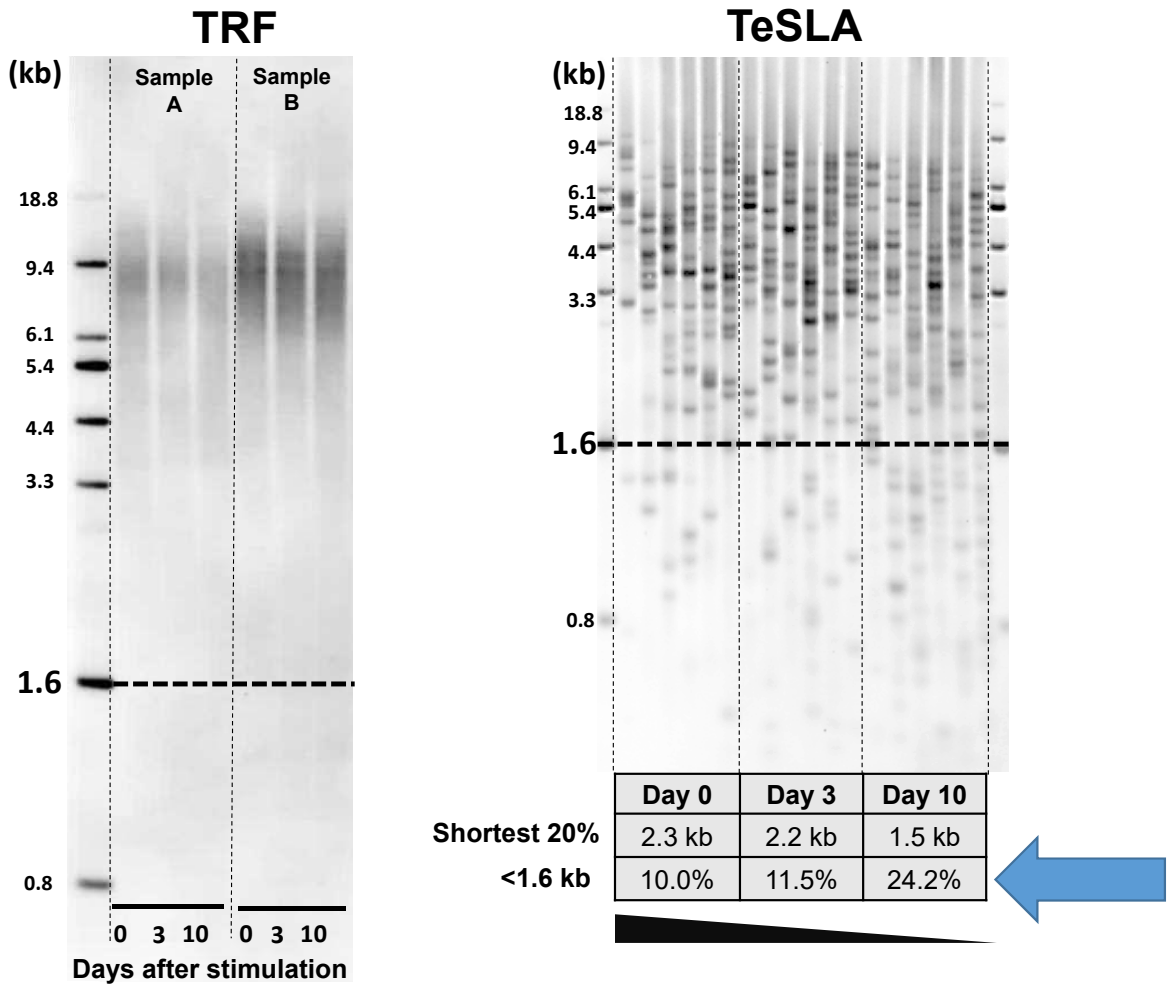
Stimulated T-Cell Responses in Centenarians



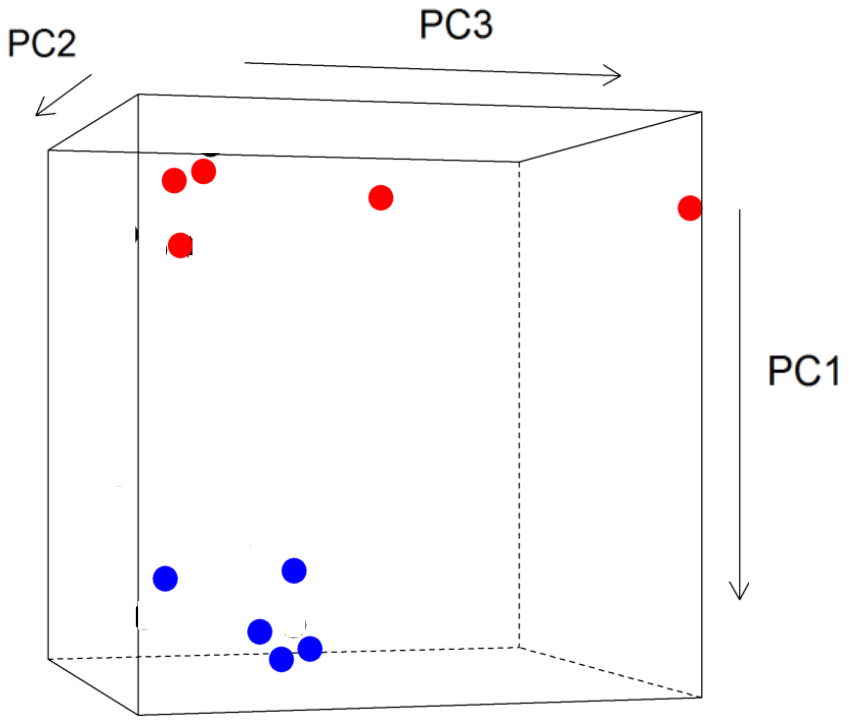




Telomere Shortening in Stimulated T-cells (10 Days)



RNA-sequencing: Principal Component Analysis (PCA) of Stimulated T-cells at Day 3 After Stimulation

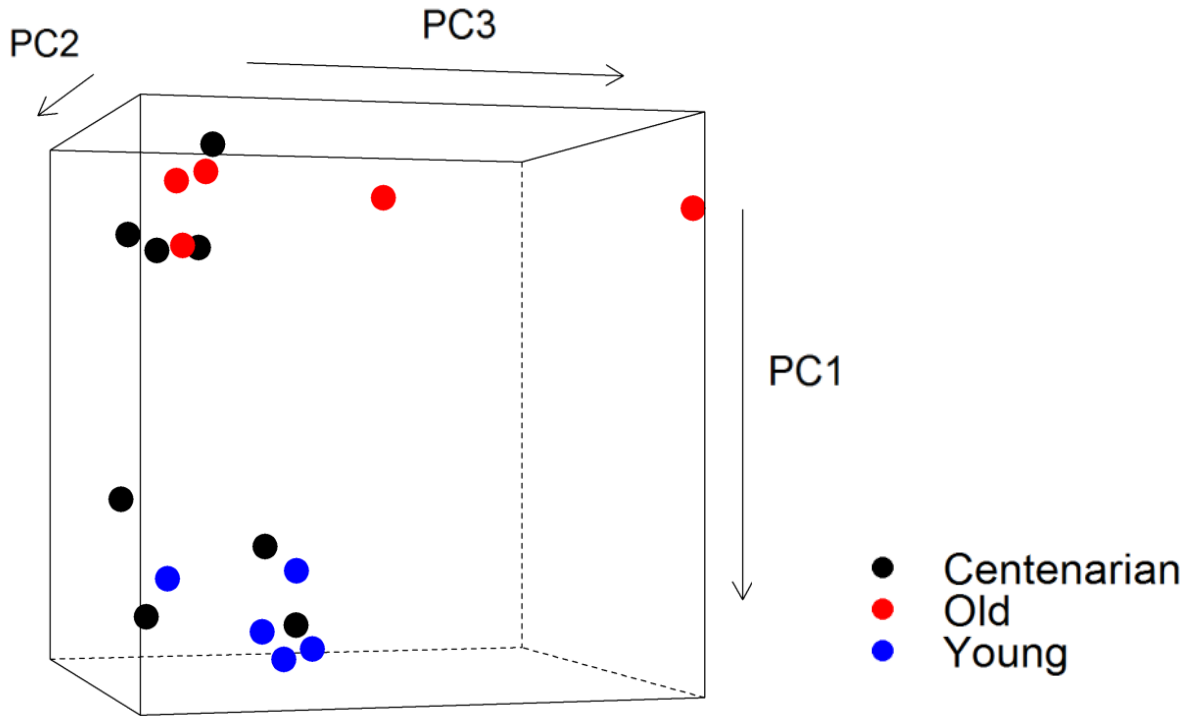


Young: 23-39 yrs old

Old: 67-83 yrs old

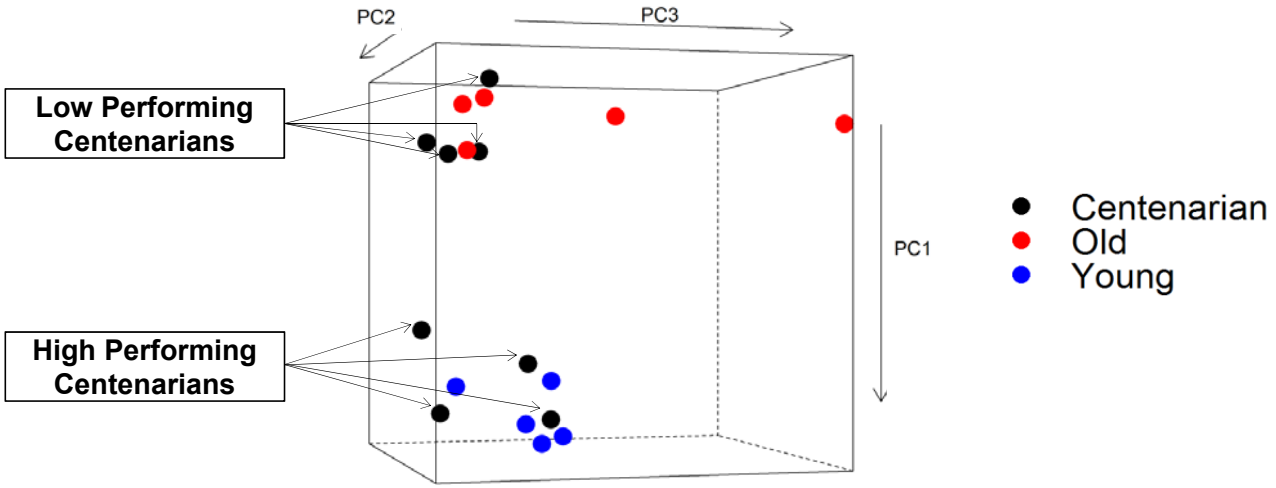
Tedone et al, *Aging Cell*, 2018

Some Centenarian Gene Expression Profiles Segregate with the Young Cohort and Others with the Old Cohort



What is the health status of the centenarians?

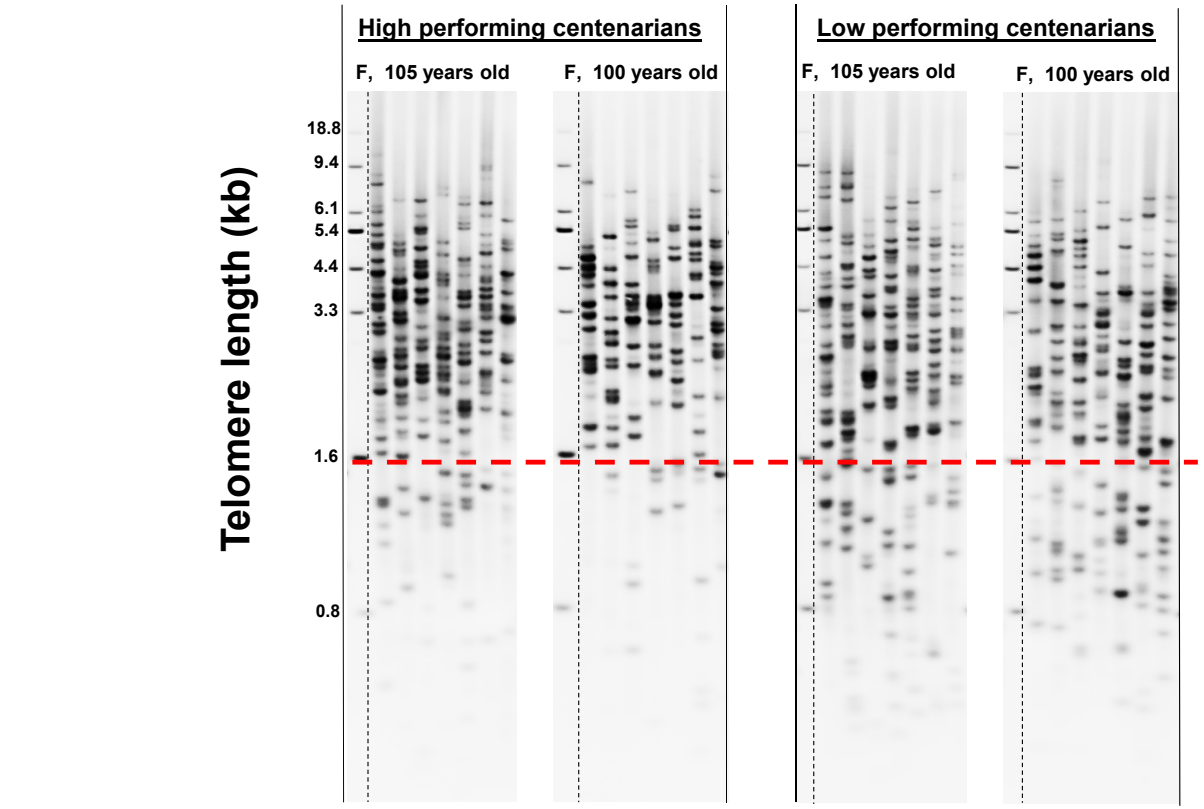
High Performing Centenarians Have a Better Health Status



Health status assessment	Low performing centenarians (n=4)	High performing centenarians (n=4)	Old subjects (n=5)	Young subjects n=5)
Age, years, mean ± S.D.	103.5 ± 3.0	104.0 ± 3.6	75.0 ± 4.2	24.5 ± 2.1
Cognitive performance, MMSE score (0-30), mean ± S.D.	14.2 ± 13.3 *	28.0 ± 1.4	30.0 ± 0.0	30.0 ± 0.0
Physical performance, IADL score (0-8), mean ± S.D.	1.8 ± 1.0 *	6.8 ± 1.5	8.0 ± 0.0	8.0 ± 0.0
Disease count per individual, mean ± S.D.	6.0 ± 0.8 *	2.5 ± 0.6	1.0 ± 0.7	0.0 ± 0.0

* $p < 0.05$ vs each of the other groups

High Performing Centenarians Have Less Critically Short Telomeres



Identification of Genes Close to Telomeres Potentially Involved in High Performing Centenarian T-cell Responses to Stimulation

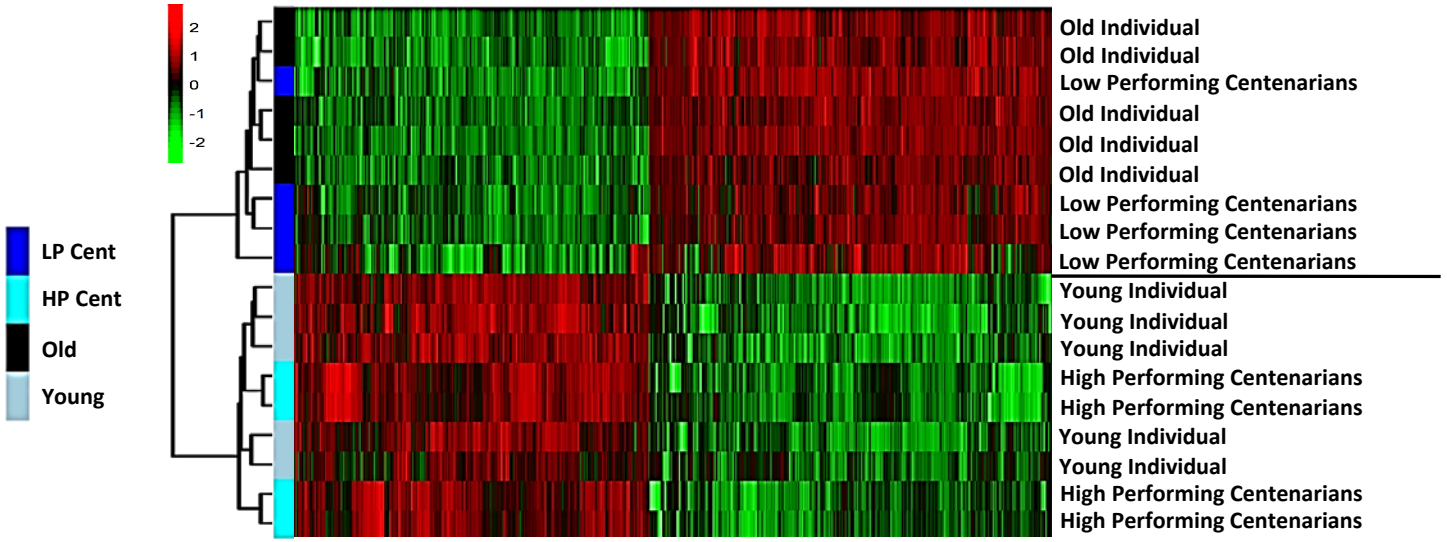
Differentially expressed genes (p<0.05) in both **(Young & HP Cent) vs Old**

∩

Genes with same directionality (fold change > 0.5) in both **(Young & HP Cent) vs Old**

↓

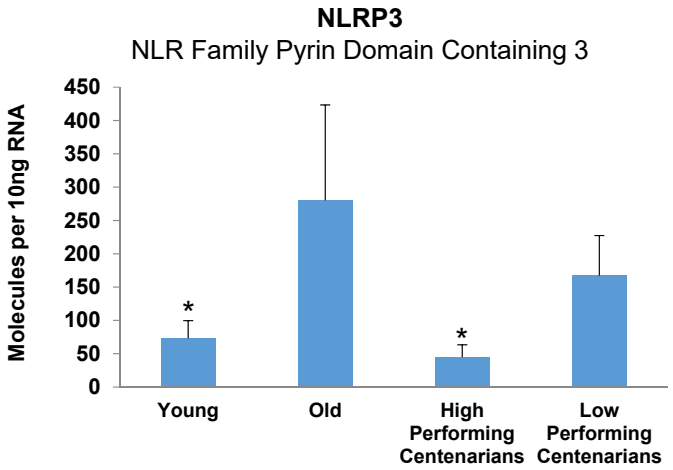
1858 Genes

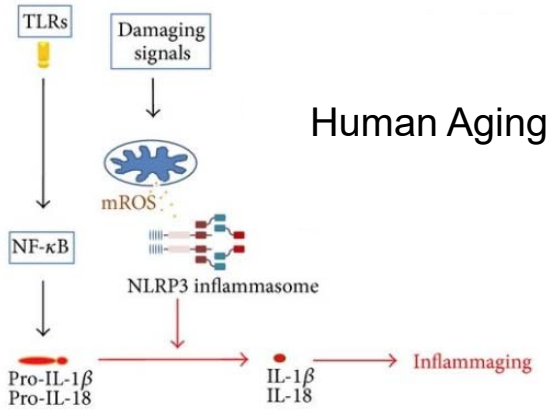


873 genes up-regulated (p<0.05) In both (Young & HP Cent) vs Old

985 genes down-regulated (p<0.05) In both (Young & HP Cent) vs Old

High Performing Centenarians Have Decreased Expression of NLRP3

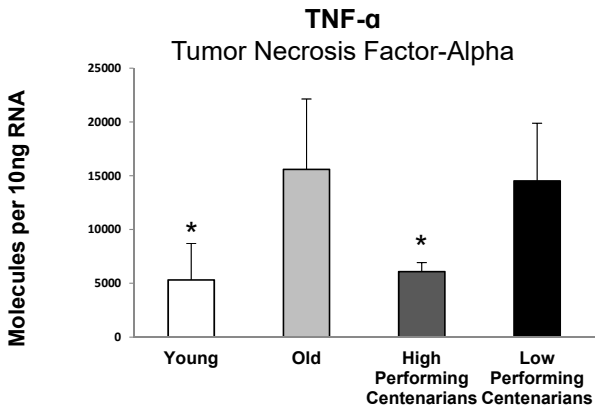


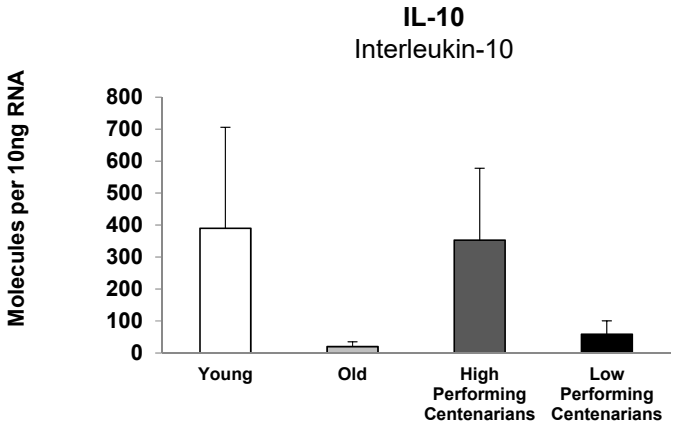


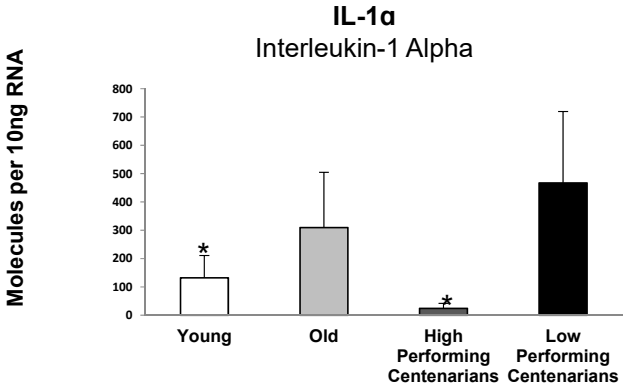
Human Aging

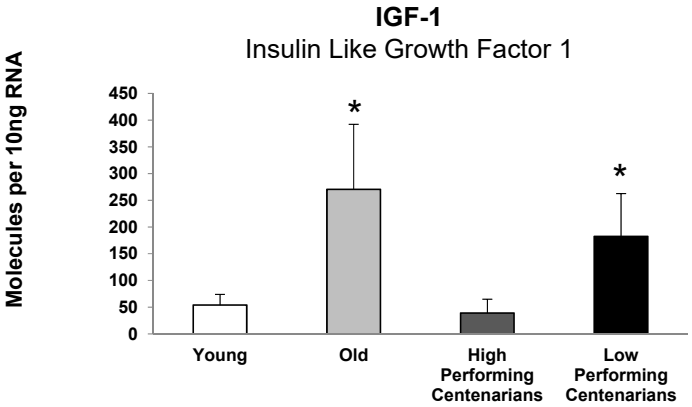
- NLRP3 is a key component of the innate immune system that induces pro-inflammatory cytokines production and cell death.
- Chromosome 1q44 ~1Mb from the telomere
- Linked to systemic low-grade inflammation and functional decline in aging
- Promotes age-related immunosenescence

High Performing Centenarians Have Expression of Growth Factors and Inflammatory Genes Similar to Younger Volunteers







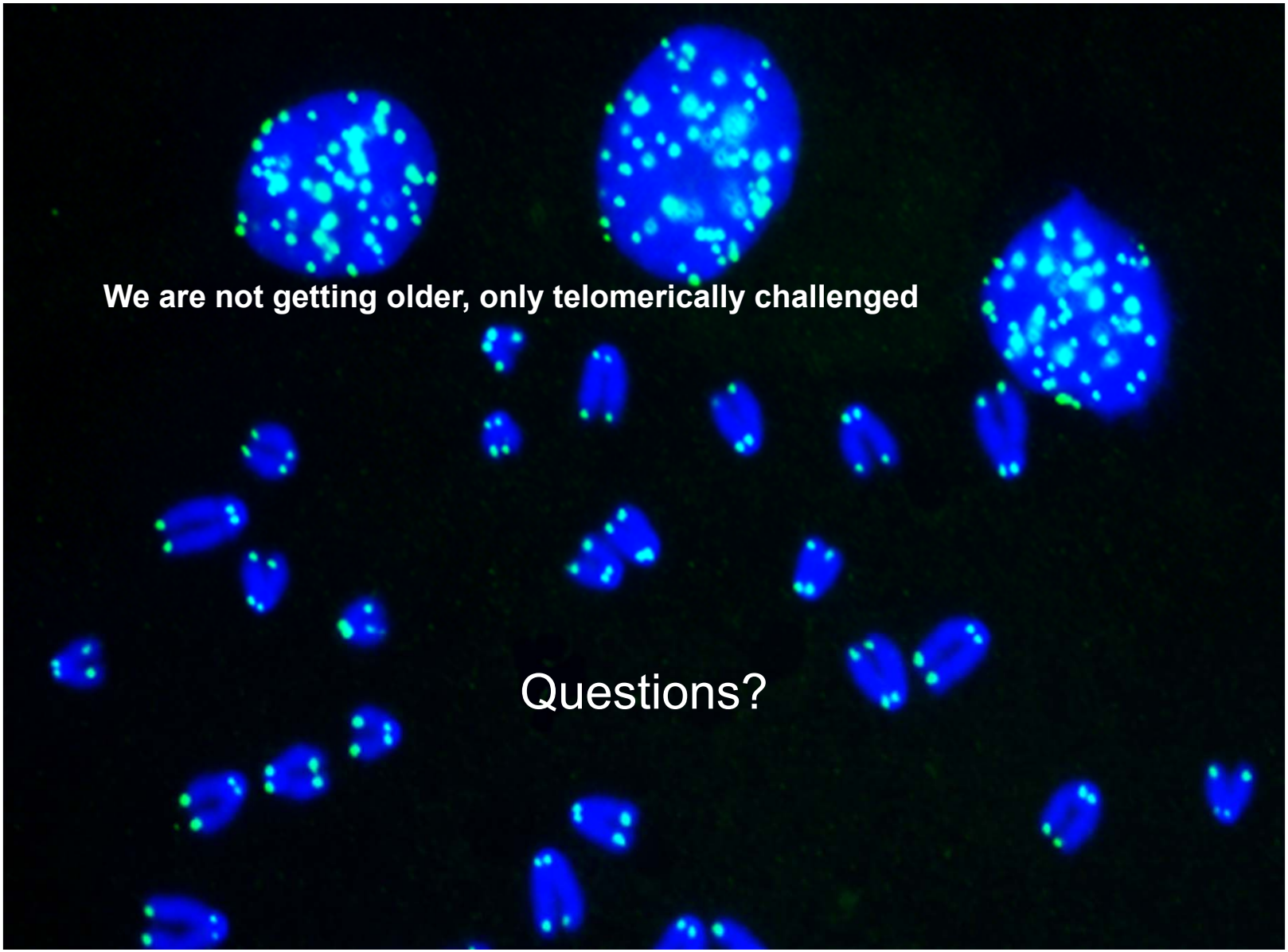


Conclusions

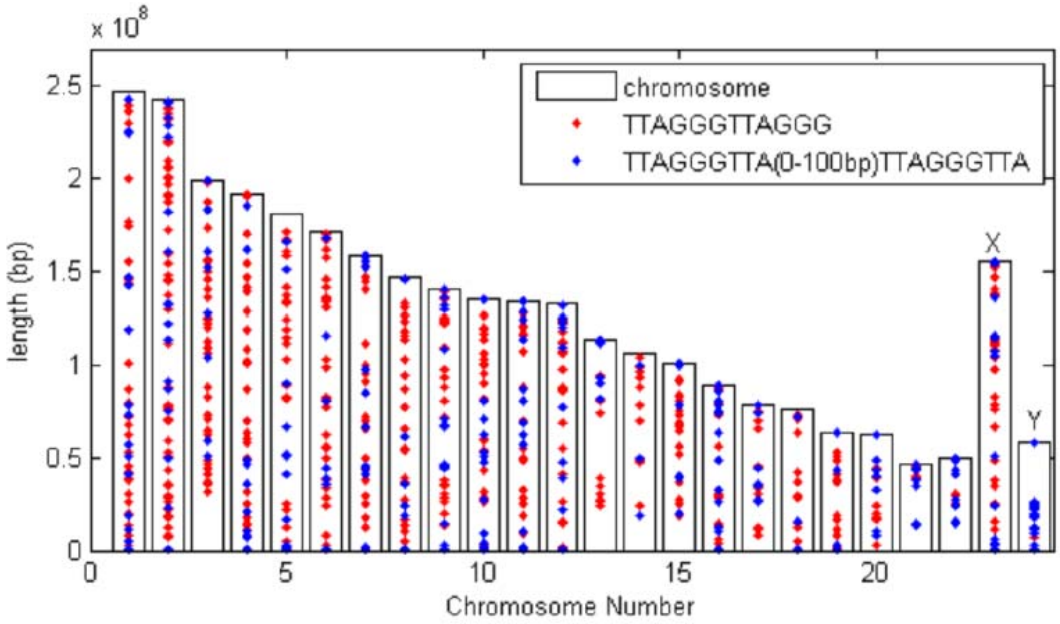
- T-cells from a subset of centenarians (high performing) have longer telomeres compared to older adults and low performing centenarians.

- Using high performing centenarians as a model of exceptional healthy aging, we have identified and validated a number of genes whose expression are potential biomarkers that may influence the risk and progression of multiple age-related conditions.

- Telomere looping (TPE-OLD) may regulate maximal human telomere length and we are beginning to understand how this may be involved in regulating gene expression in aging and disease onset.

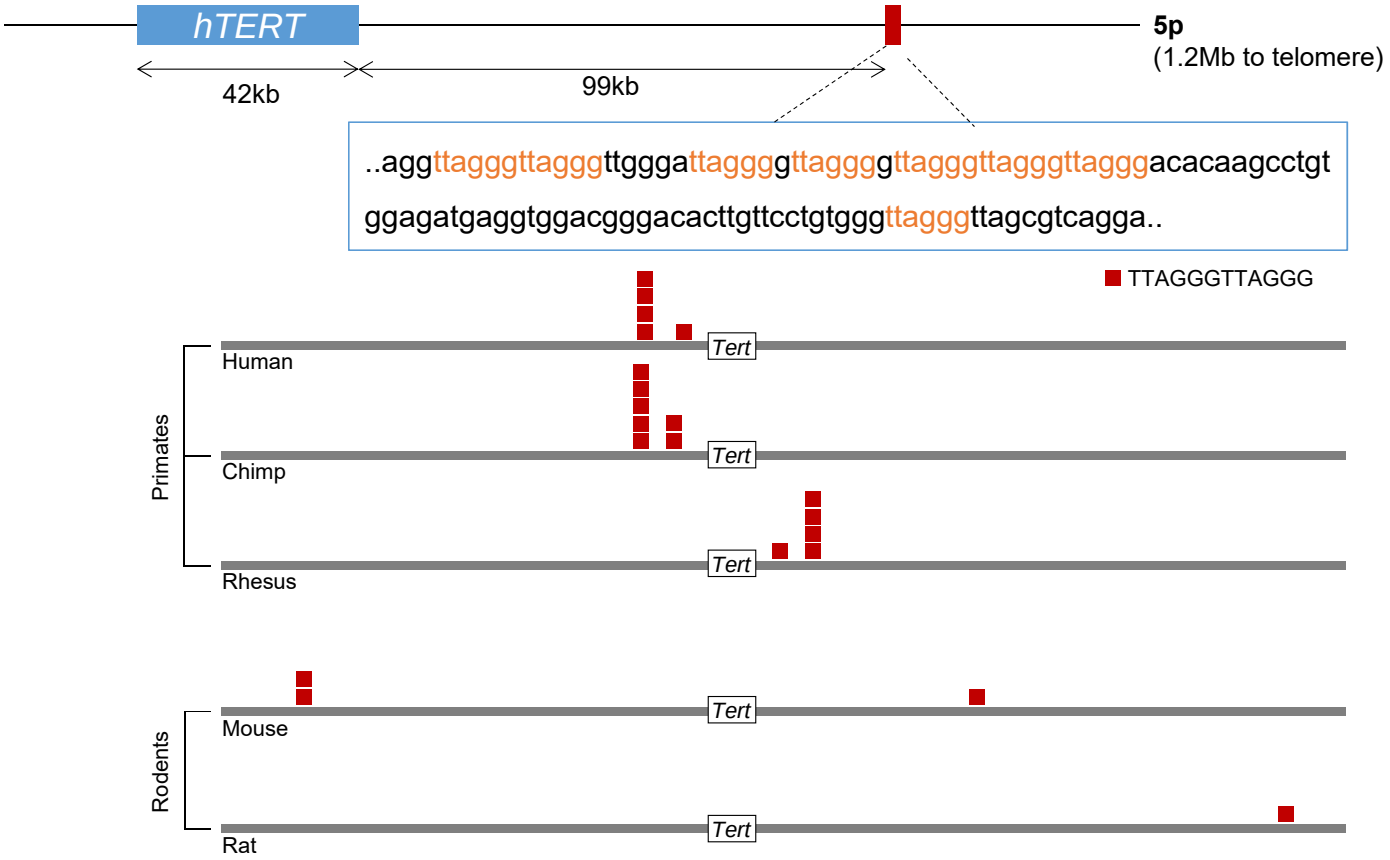

We are not getting older, only telomerically challenged
Questions?

2920 Interstitial Human Telomere Repeats

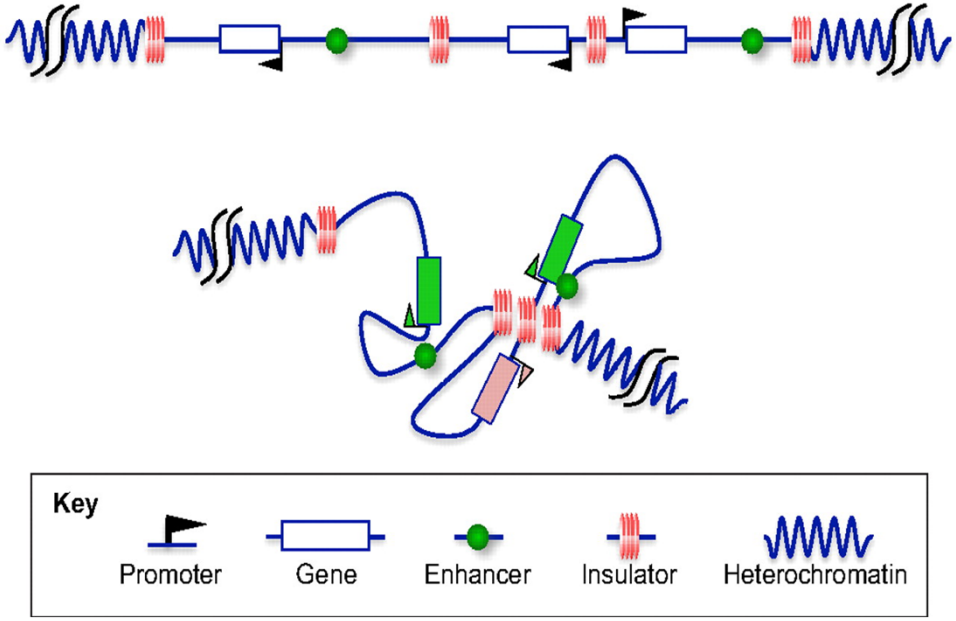


Wood et al Nature Communications, 5:5467 doi:10.038/ncomms6467 (2014) Steve Kosak lab

There are Telomeric Interstitial Repeats Close to the Primate TERT Locus

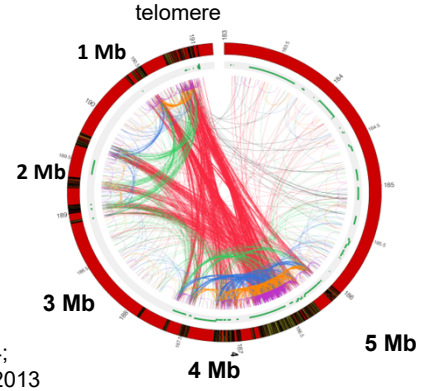


Activating and Repressing Transcriptional Events by Chromosome Looping



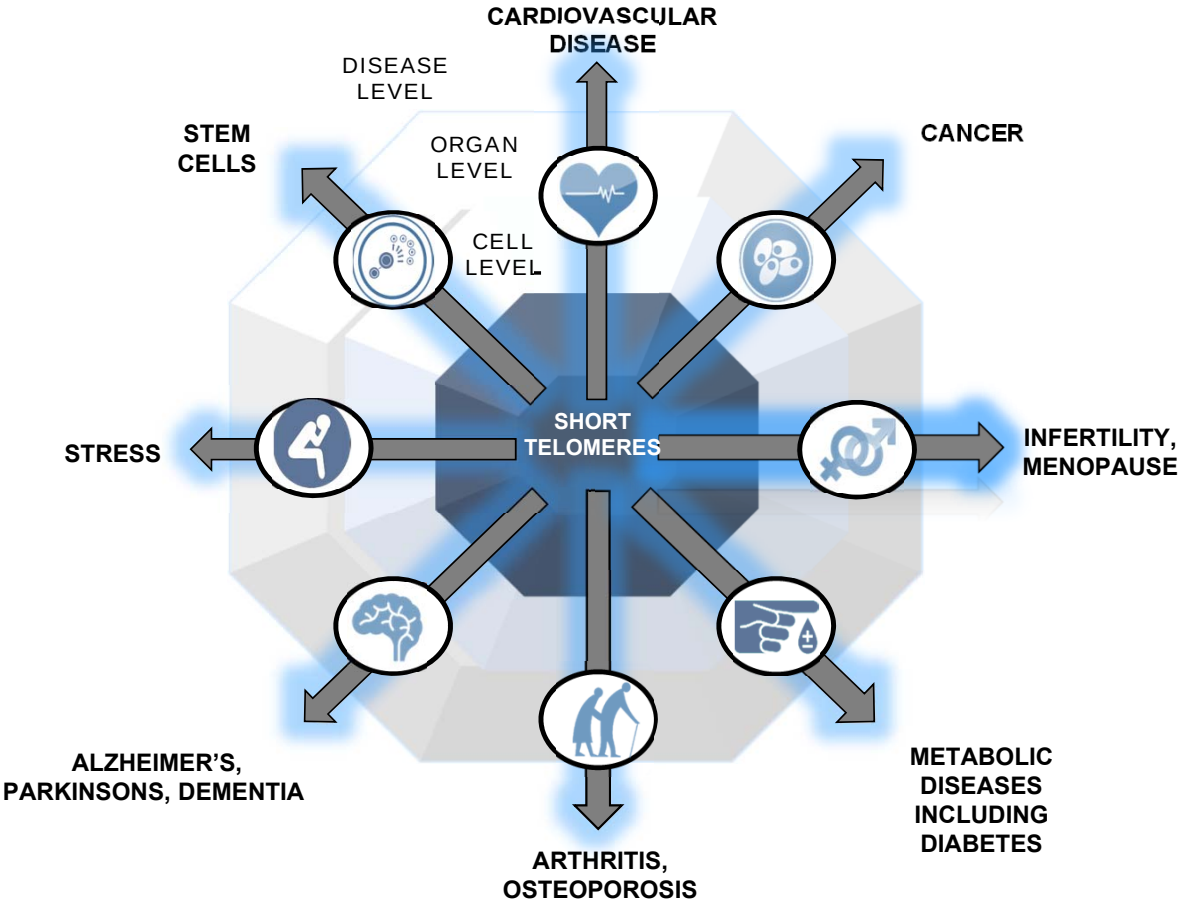
Job Dekker lab *Dev*. 139: 1045, 2012

We modified the Circularized Chromosome Conformation Capture (4C) method and used 3D co-FISH to determined if genes near telomeres are regulated by chromosome looping at the telomeres



Robin et al, *Gene Dev* 2014;
Stadler et al *Nature Comm* 2013

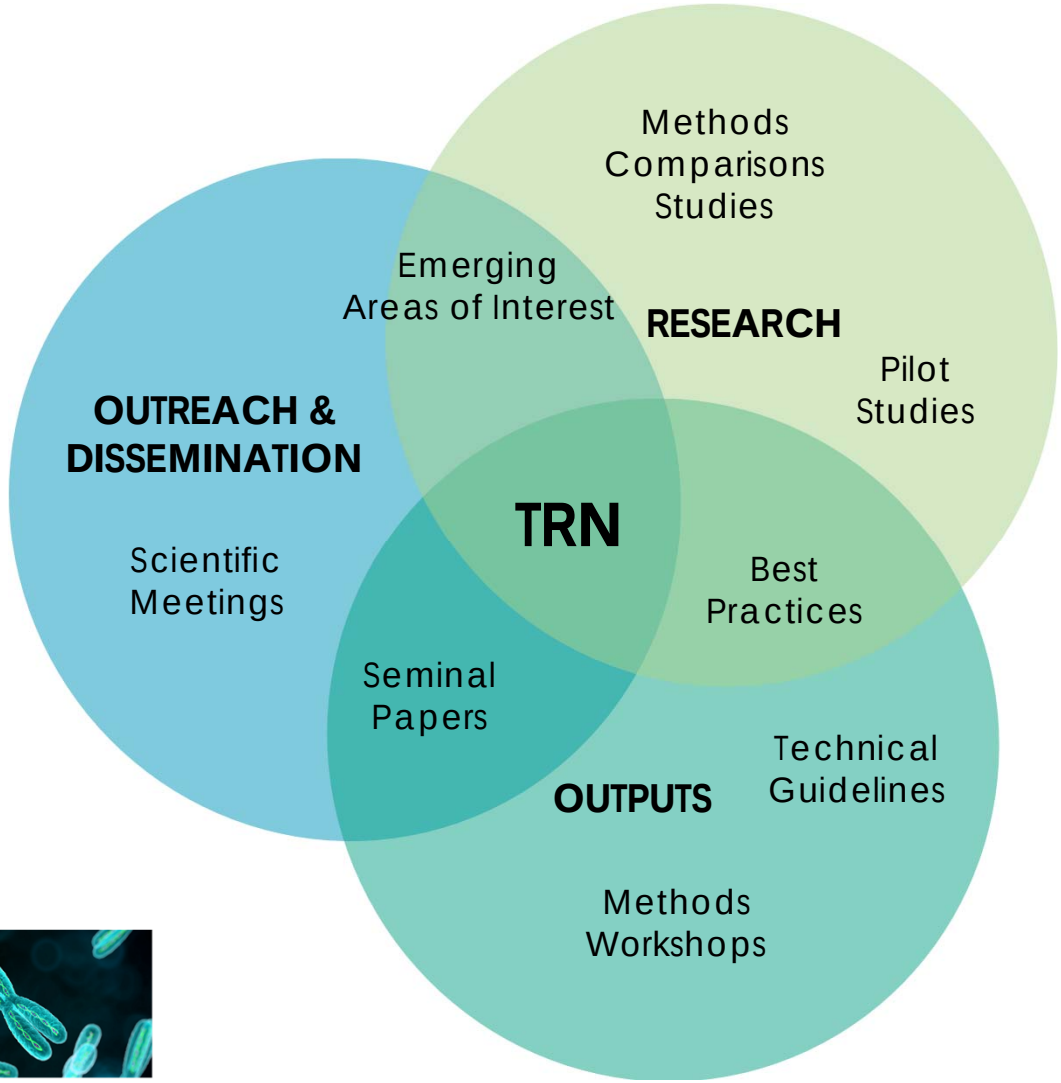
Short Telomeres Correlate With and Potentially Contribute to Everything. Really?



Telomere Research Network Roadmap

NIH NIEHS

NIH NIA



TELOMERE
RESEARCH
NETWORK

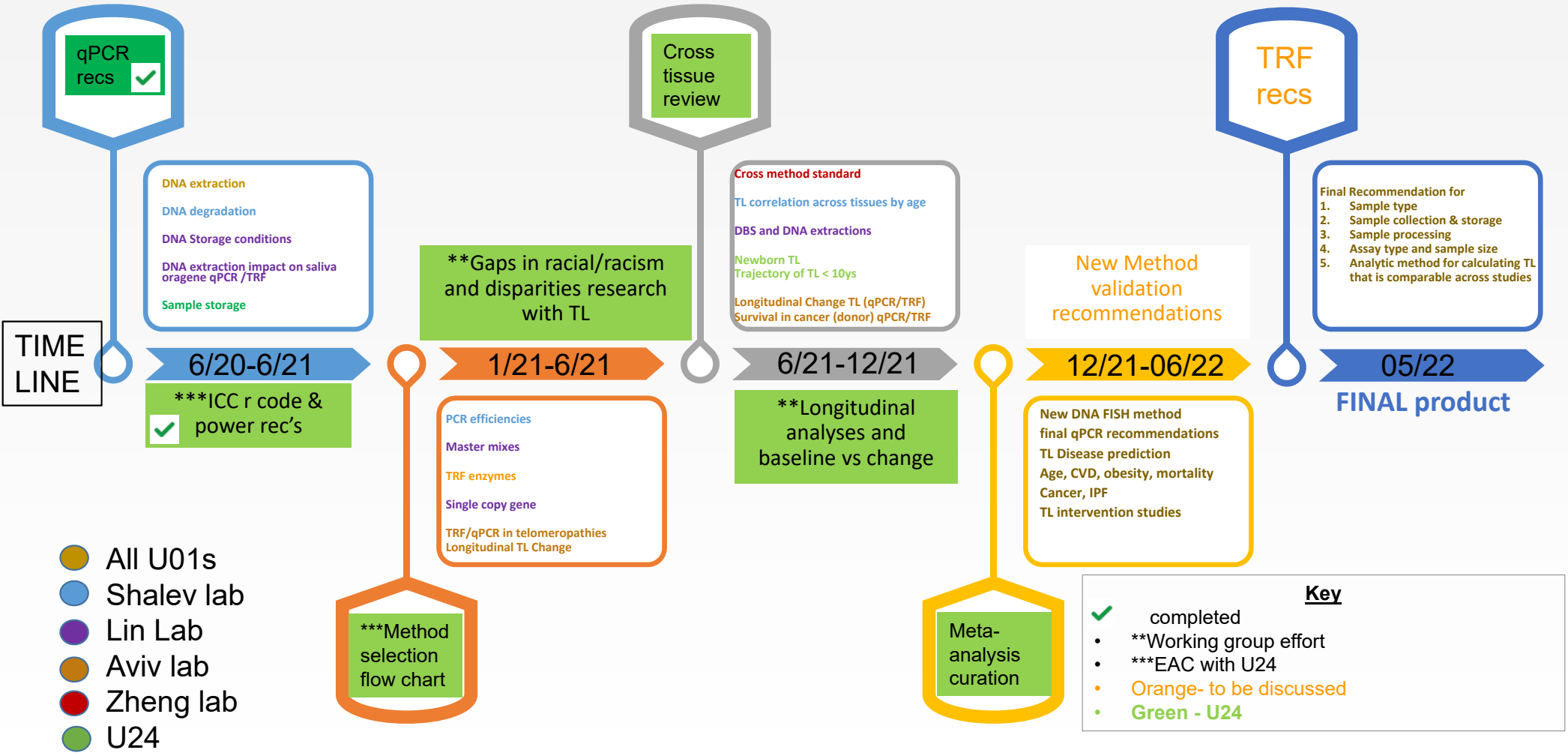
Two parts of the TRN

- U01s (yr 1-3)
  - Methods comparison studies
    - qPCR, TRF, FISH, WGS, others….
  - Impact of preanalytic factors (Lin)
  - Cross tissue correlation (Shalev)
  - High throughput qFISH TL assay (Zheng)
  - Clinical utility of TL in cancer, transplantation, and telomere syndrome disorders (Aviv)

- TRN
  - Disseminate best practices
  - Develop a cross disciplinary interactive network
  - Define the applicability of telomere length as a marker of psychosocial and environmental stress and a predictor of disease
  - Support innovative research efforts focused on key questions

TELOMERE RESEARCH NETWORK

YEAR 1 to 3:cross method timeline

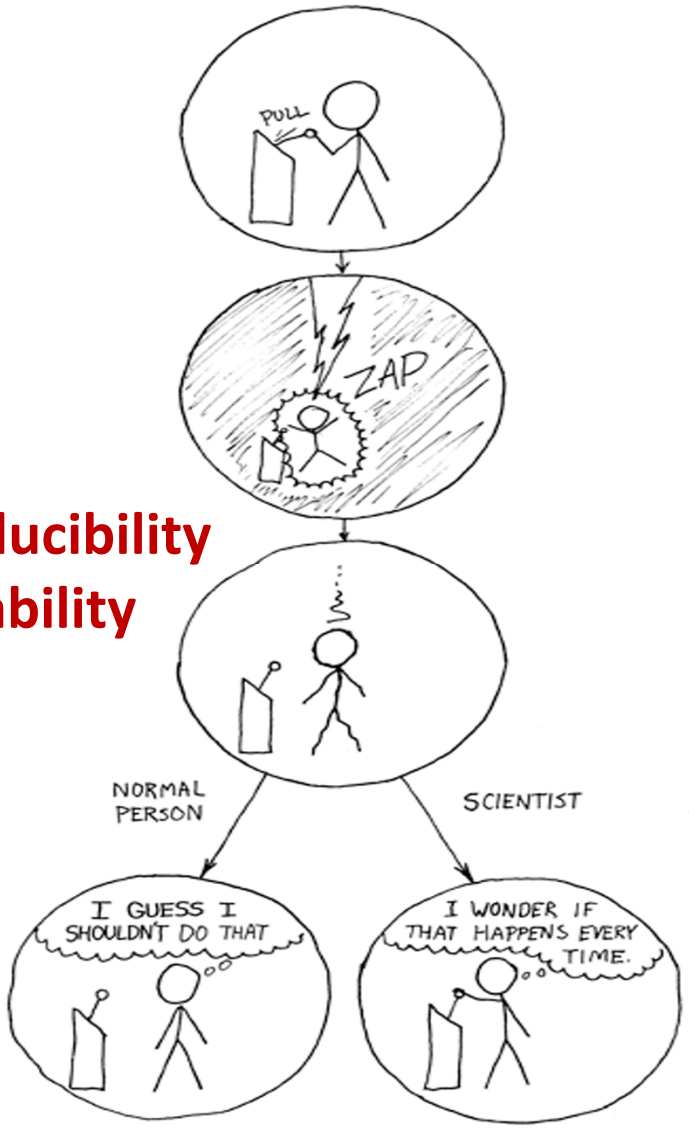


Systematic and rigorous testing of key methodologic considerations to provide empirical support for reporting

- Evaluate impact of pre-analytic factors
  - Extraction, storage across sample types used in population based studies
    - Peripheral blood, saliva, buccal, dried blood spots
- Evaluate the impact of variability in assay conditions routinely used
  - qPCR
    - Primers- single copy, master mixes, PCR efficiencies,
  - TRF
    - Restriction enzymes
- Evaluate the correlation across TL measured in different peripheral tissues across development
- Evaluate the differential ability of assays to predict disease

One goal- IMPACT

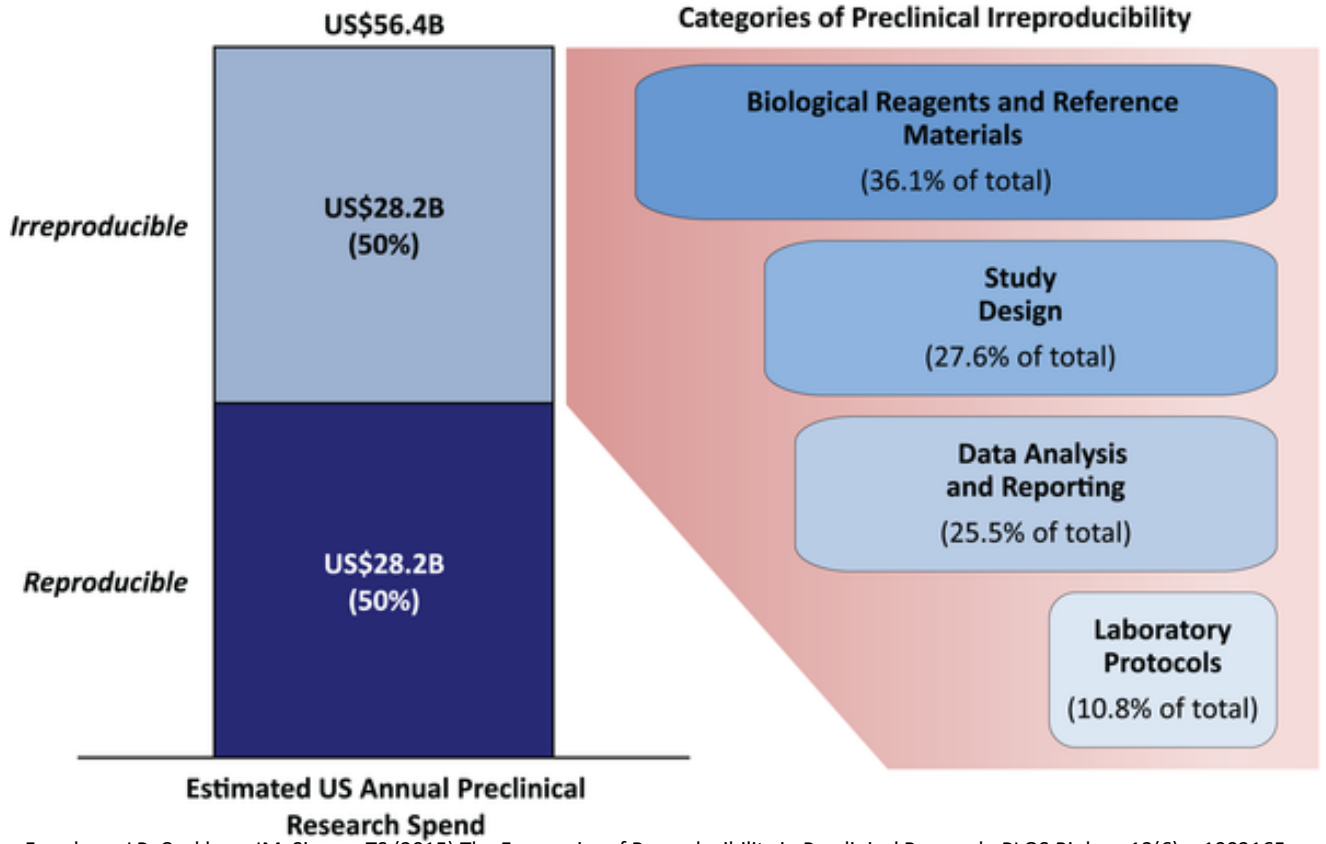
Reproducibility
Replicability



Reproducibility and Replicability

TELOMERE
RESEARCH
NETWORK

Fig 2. Estimated US preclinical research spend and categories of errors that contribute to irreproducibility.



Freedman LP, Cockburn IM, Simcoe TS (2015) The Economics of Reproducibility in Preclinical Research. PLOS Biology 13(6): e1002165. https://doi.org/10.1371/journal.pbio.1002165
https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1002165

The TRN toolbox and research support

Tools on the web

- Protocols for TL measurements
- qPCR reporting guidelines
- R-code for ICC calculations
- Power calculations for sample size with identified ICC
- Telomeres in health and disease primer
- **Seminal telomere length papers**
- **Methodology selection flow chart**
- **Meta analyses curation**

Support and consultation

- Current
  - Consultation with experts
  - Connections with TL laboratories
  - Listserve for events and RFAs
- Developing
  - Methodology selection flow chart
  - Subcommittees
  - Quarterly research highlights
- Coming soon
  - Telomere length measurement workshops

TRN Lab Protocols

qPCR TL Assay Protocols

U01 Shalev: Absolute TL

U24: MMqPCR

TRF TL Assay Protocols

U01 Aviv: TRF Protocol

U01 Aviv: Data Analysis

qFISH TL Assay Protocols

U01 Zheng

Sample Collection/Storage

U01 Lin: Blood Collection for TL Measurement

U01 Lin: DNA Aliquoting and Shipping

DNA Extraction Protocols

U01 Lin: DNA Extraction from Whole Blood

U01 Lin: DNA Extraction from DBS

U01 Lin: DNA Extraction from Saliva

U01 Shalev

Study design
- New study
- Archived
- Secondary data analyses

Sample type
- Fresh live cells
- Archived/nonviable

Cohort size
- Small < 300
- Moderate 300-600
- Large >600

DNA amount
- <1 ug
- >1ug

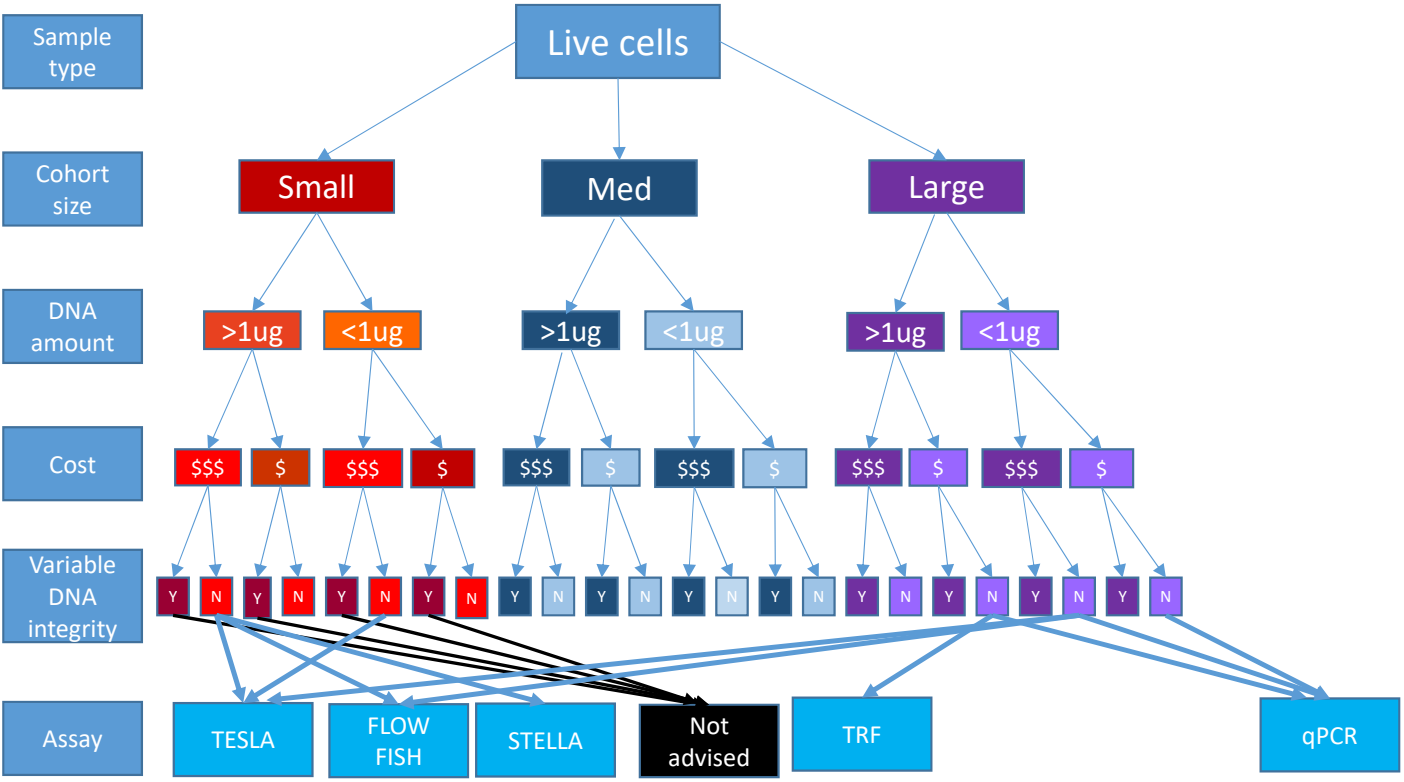
Cost
- >40/sample
- <40/sample

DNA integrity concerns
- Yes- major
- No- minor

Method selection tool:
Redcap link (planned) to guide researchers in deciding what/if TL measurement appropriate

TELOMERE RESEARCH NETWORK

New Study with live cells- EXAMPLE 1



TRN subcommittees

- Epidemiology
  - Belinda Needham, Chair
- Health Disparities
  - Carmen Giugescu and Dawn Misra, Co- Chairs
- Cancer
  - Ling Zhang and Shahinaz Gadalla, Co-Chairs
- Early Development
  - Sonja Entringer, Chair

- Coming soon
  - Aging
  - Environmental Exposures
  - Pulmonary diseases

U24/TRN goals

Year 2

- 2nd round of pilot awards
- Free method selection on-line tool
- Quarterly Telomere studies e-news letter
- Development of Aging and Environmental exposures subcommittees
- Manuscript of predictors of newborn TL
- Meta analyses/systematic review of relation between racism/social determinants of health and TL
- Summer/Fall 2021 TRN meeting (European location, TBA)

Year 3

- 3rd round of pilot awards
- TRF reporting guidelines
- Interactive telomere researcher database
- Empirical data on TL trajectory across tissues for first decade of life
- TRN projects RFA for year 4
- Protocol for telomere measurement workshops

TRN topic webinars

- Available via TRN- email telomerenetwork@gmail.com
  - Introduction to the TRN- July 2020
  - Telomeres and COVID- August 2020

- UPCOMING
  - Health Disparities/Social Determinants
    - TBA- January 2021
  - Telomere length as a clinical and prognostic indicator- focus on idiopathic pulmonary fibrosis
    - TBA – March 2021

PATIENT CARE

RESEARCH

TEACHING

SERVICE

Georgetown | Lombardi

COMPREHENSIVE CANCER CENTER

New Method Development for TL Measurement: An Update

Yun-Ling Zheng

Georgetown University Medical Center

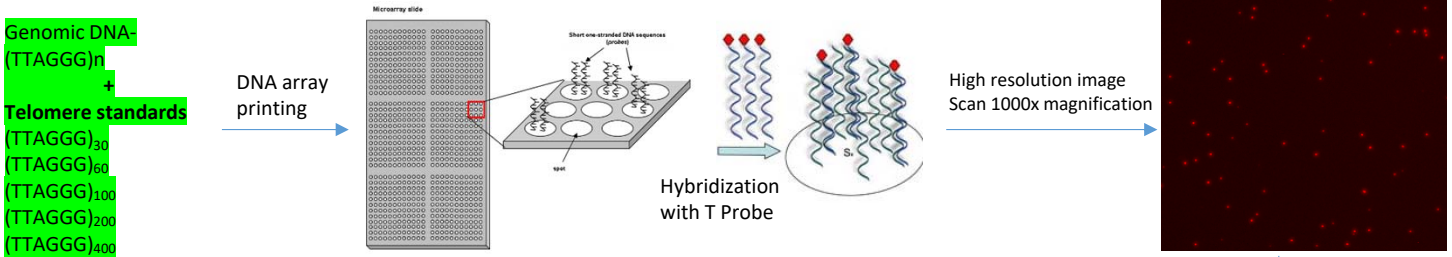
NCI Comprehensive Cancer Center

A Cancer Center Designated by the National Cancer Institute

http://lombardi.georgetown.edu

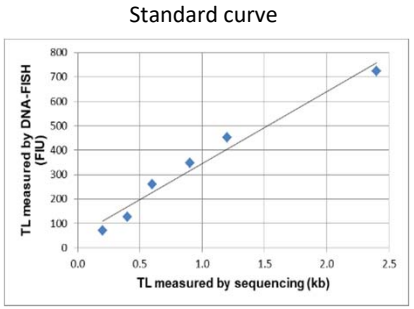
Lombardi CancerLine: 202.444.4000

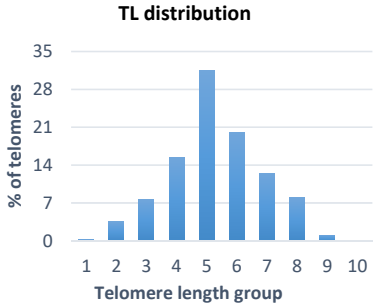
Schematic Overview of the DNA-FISH Single Telomere Assay Workflow



Dot #	TL (FIU)
1	550
2	3867
3	9725
4	9099
5	7391
6	11944
7	6860
8	3582
9	1391
10	4662

Convert FIU into kb





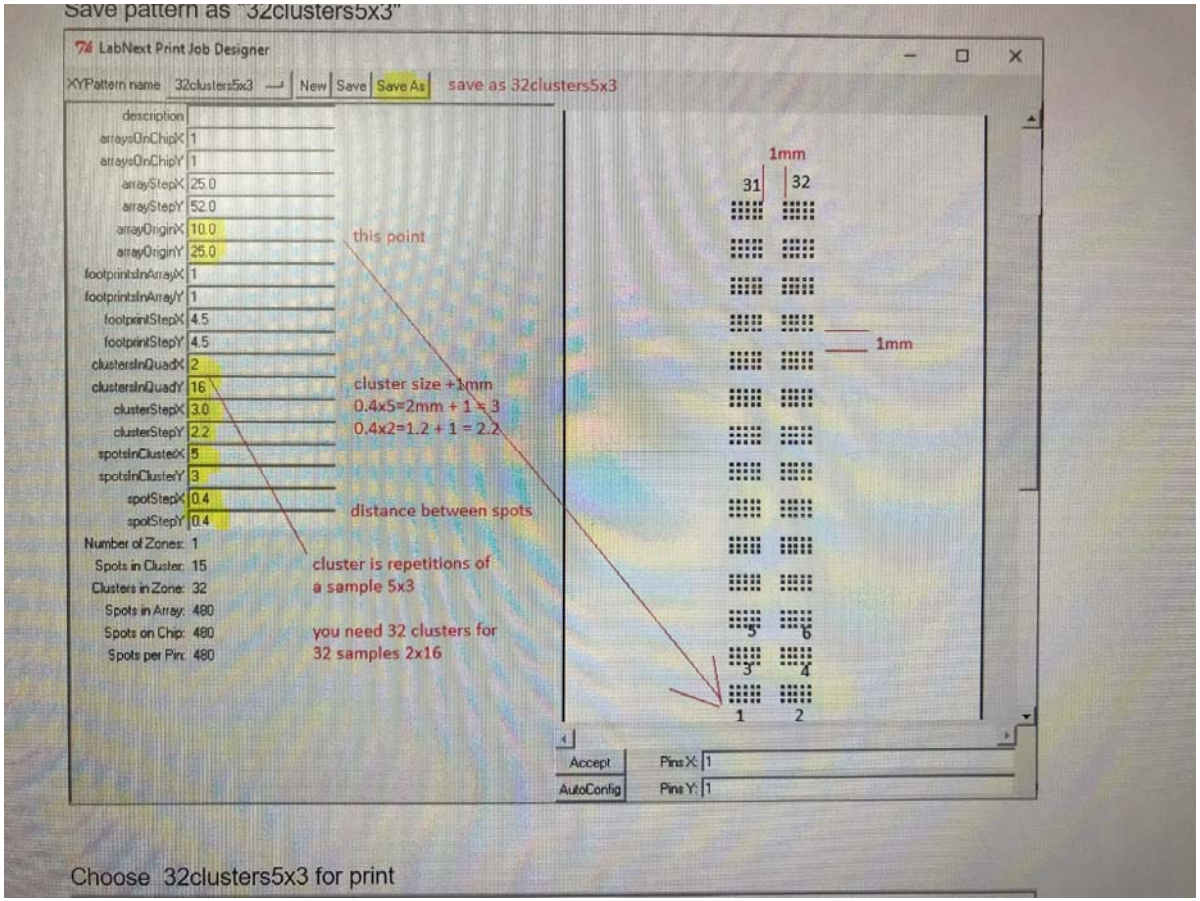
TL = telomere length, FIU = fluorescent intensity unit
DNA array was hybridized with Cy-3 labelled telomere probe $(TTAGGG)_3$

DNA-FISH Single Telomere Assay

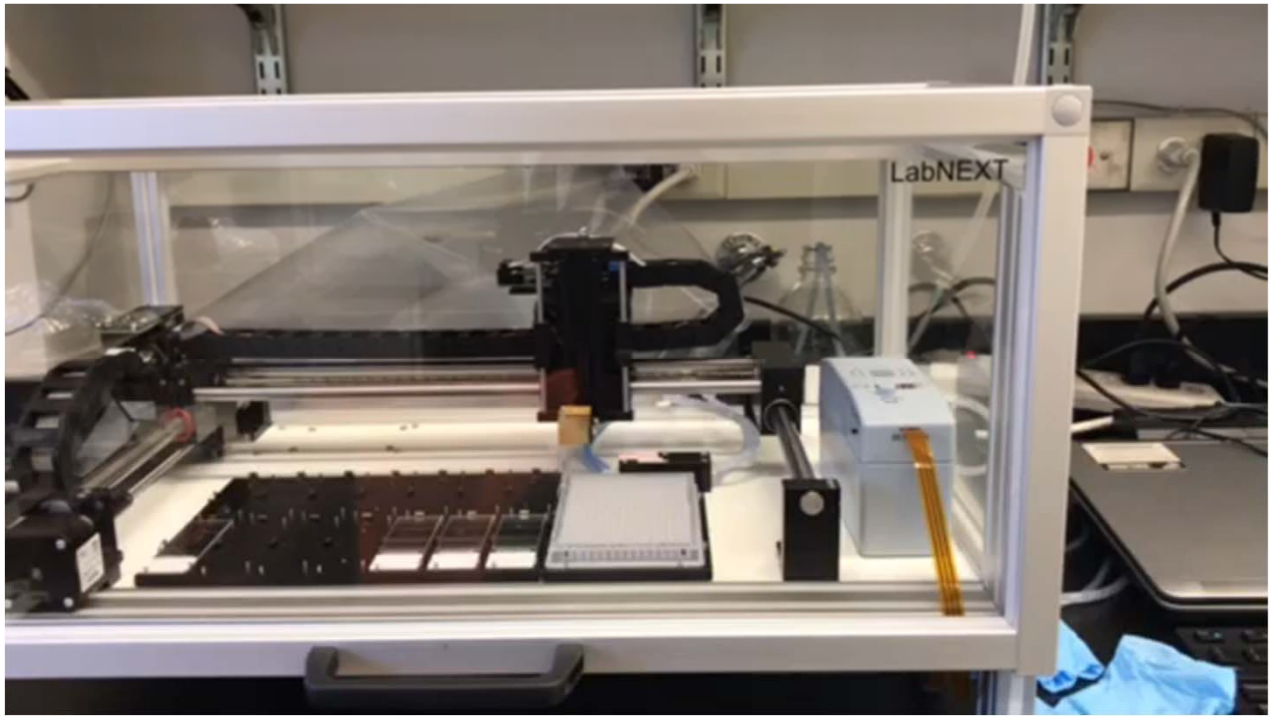
Main tasks to achieve high-through-put:

1. Print high quality DNA array
2. Molecular cloning of telomere fragments as size standards
3. Full automation of array scanning and image analysis

Array design: 5x3=15 dots per cluster; 20 samples per row and 6 samples per column = 120 samples per slide.



Two slides will be printed per sample sets.
LabNEXT's Xactii prints 384 samples each run

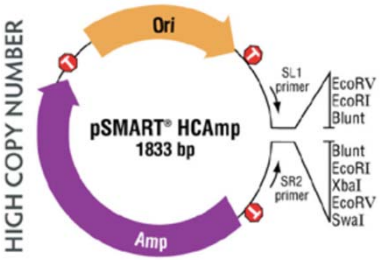


Cloning Telomere Fragments as Size Standards

- Molecular cloning of telomere fragments starts with synthetic T fragment of 100 bp.
- Through repeated cloning and extension, we obtained clones containing 0.1 – 4.8 kb T fragments.
- Clones contain > 2.4 kb T fragment are not stable, suffered insert alteration/deletion during subsequent cultures.
- Vectors tried: pUC19, pBR322, pSMART, pBluscript KS, pJAZZ.
- Currently stable clones include: 0.1, 0.2, 0.4, 0.6, 0.9, 1.2, 1.5 and 2.4 kb.

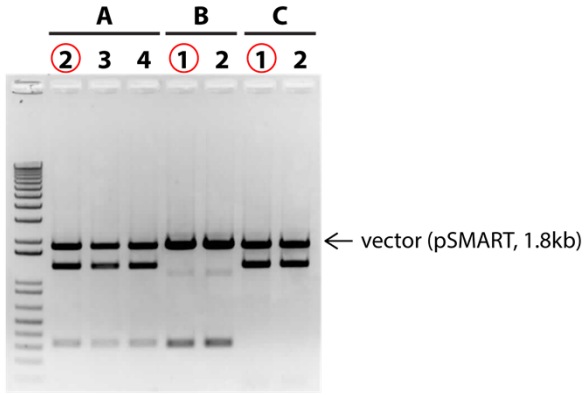
Alternative telomere construct.

- Changed vector backbone to pSMART
- More information, see https://www.lucigen.com/



pSMART® Vectors
All pSMART vectors are the unique design of these vectors eliminates transcription into and out of the insert DNA, reducing cloning bias. Strong promoters driving indicator genes or negative selection genes (*lacZα* or *ccd*B) can cause plasmid instability with their strong secondary structure, and by transcription of toxic inserts. pSMART vectors do not contain a promoter or indicator gene, so transcription across the insert is avoided. Strong transcription terminators flank the insert cloning site to eliminate fortuitous transcription from cloned inserts.

Enzyme digestion



A: HBB + Adaptor + Telomere (~1350 bps + ~350 bps)
B: HBB only (~350 bps)
C: Adaptor + Telomere (~1350 bps)
Each clone digested with EcoRV

HBB+ADP+TLMR in KS was digested by KpnI+SacII and fragment was further treated with Klenow fragment to make it blunt end.
Digested blunt-end fragment was ligated in pSMART vector (A2-4, **HBB+ADP+TLMR in pSMART**).
Clone B1 and C1 were generated using generated clone A2.

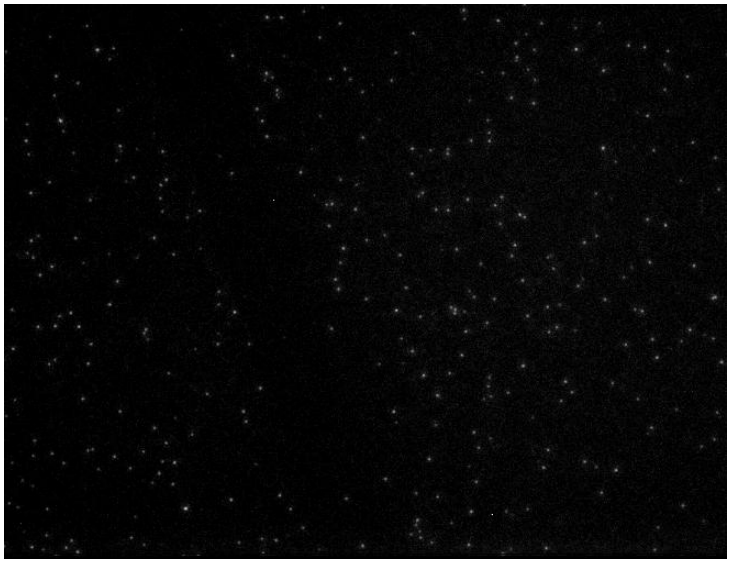
ADP and TLMR were removed by digestion of BamHI+HincII, then blunt-ended and performed self-ligation (B1-2, **HBB only in pSMART**)
Finally, HBB was removed by digestion of NotI+BamHI, then blunt-ended and performed self-ligation (C1-2, **ADP+TLMR in pSMART**).

Automation of Array Scanning and Image Analysis

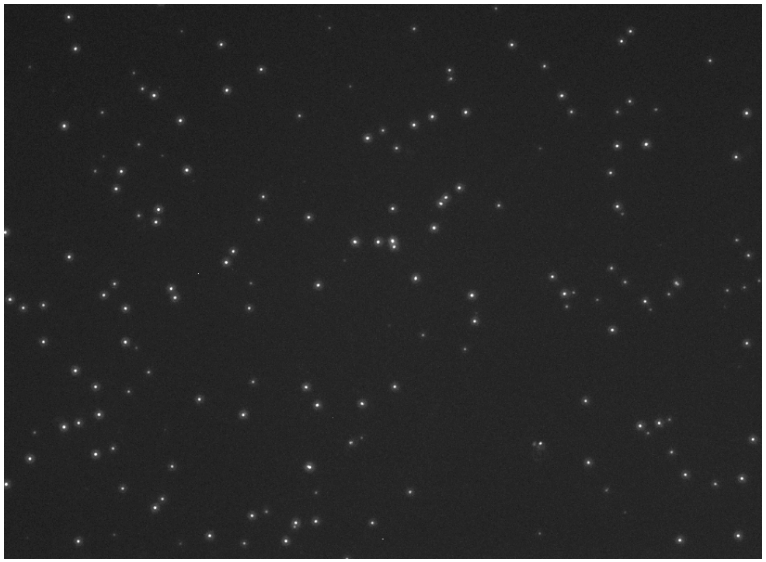
- Molecular Device: ImageXpress Nano or Micro 4.
- Leica microscope and image system.
- MetaSystems: Metafer and ISIS.

DNA FISH of telomere standards using the PNA telomere probe

0.4 kb telomer clones



2.4 kb telomere clones



Telomere DNA FISH of U2OS genomic DNA

U2OS is a cancer cell line has long and heterogonous telomere length

Acknowledgement

- Georgetown University Medical Center

Ying Wang, MB/BS

Bing Sun, PhD

- University of Oklahoma Health Science Center

Yusuke Takahashi, PhD

Impact of cell type on DNA integrity, and efficiency approach and uninterruptible power supply on qPCR estimates

Idan Shalev, U01 PI, Penn State University

Key personnel: Waylon Hastings, Nilam Ram, Sue Siegel

PENNSTATE 1855

U01: The Comparability and Reproducibility of Telomere Length Measurements

- **Aim 1: Comparability of TL across commonly sampled tissues, from birth to age 75 years**

Table 1: Summary of tissues/samples for Aim 1

Sample	Age (years)	Collection points	Whole blood	PBMC	DBS	Cord blood	Saliva	Buccal cells
Adults (N=100)	18-75	1		X	X		X	X
Children study 1 (N=100)	8-13	1	X		X		X	X
Children study 2 (N=100)	5-15	1					X	X
Children study 3 (N=100)	16	1					X[1]	X
Mothers (N=100)	18-43	2	X				X	X
Infants/toddlers (N=100)	Birth-3	5			X	X (N=62)	X	X

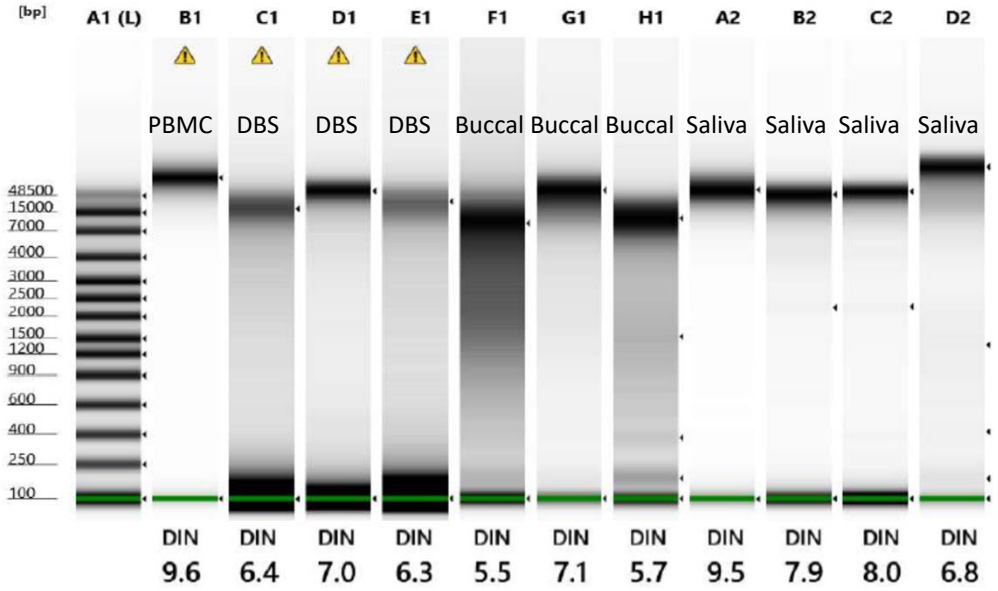
[1]Saliva x 2 (Oragene and passive drool).

Progress and next steps

- Ongoing recruitment of participants (Aim 1)
  - So far 77 participant completed the study

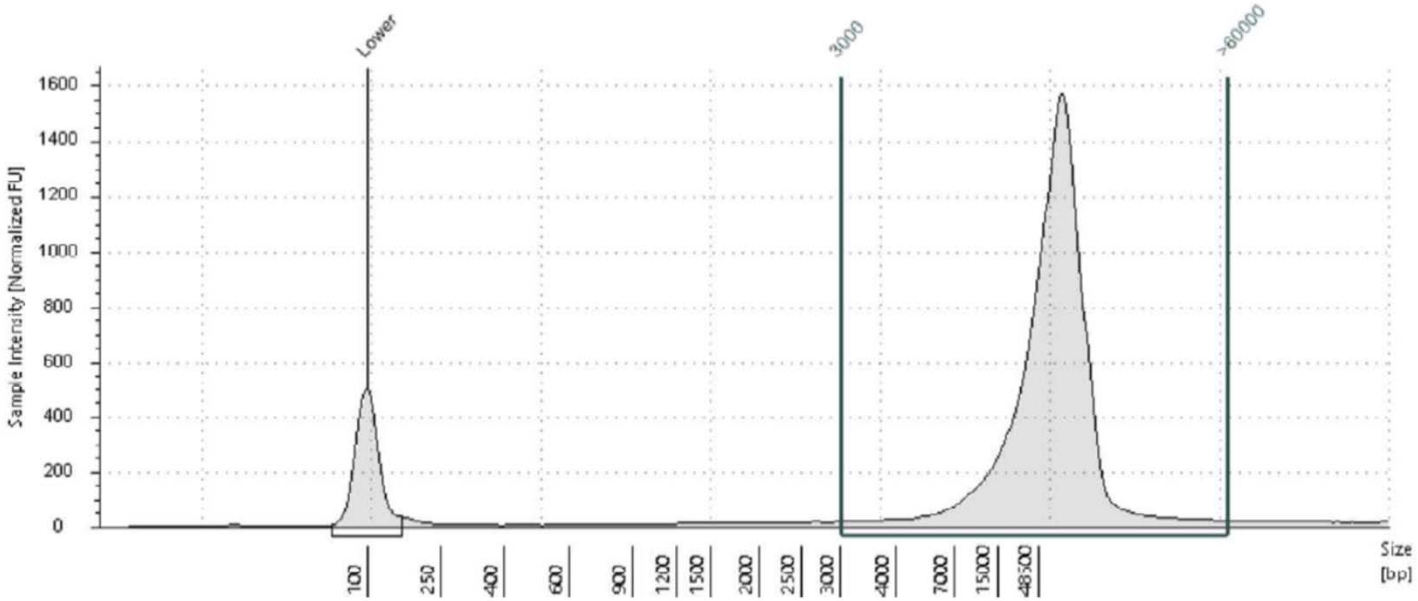
- Focusing first on DNA extraction tests and DNA QC (NanoDrop 2000 spectrophotometer, Quant-iT PicoGreen dsDNA Assay, and Agilent TapeStation bioanalyzer), followed by qPCR assays (aTL, T/S), TRF and potentially other methods

DNA integrity: TapeStation bioanalyzer



Lane	Sample ID	Nucleic acid concentration	260/280	260/230	DIN	Highest size (Bp)	% region 3kb-60kb	Tape Station Concentration
B1	TL-02 PBMC	230.90 ng/uL	1.87	0.83	9.6	>60000	94.04	102.00 ng/uL
C1	TL-01 DBS	27.10 ng/uL	1.64	0.34	6.4	17711	87.94	4.27 ng/uL
D1	TL-02 DBS	26.60 ng/uL	1.53	0.24	7	57439	88.1	5.45 ng/uL
E1	TL-03 DBS	27.83 ng/uL	1.53	0.28	6.3	23638	70.24	3.33 ng/uL
F1	TL-01 Buccal	377.27 ng/uL	1.76	0.98	5.5	9623	92.27	85.10 ng/uL
G1	TL-02 Buccal	608.83 ng/uL	1.8	0.85	7.1	58278	68.42	69.50 ng/uL
H1	TL-03 Buccal	239.50 ng/uL	1.75	0.67	5.7	11767	55.98	66.90 ng/uL
A2	TL-01 Oragene Saliva	156.93 ng/uL	1.84	1.06	9.5	59775	44.5	55.30 ng/uL
B2	TL-03 Oragene Saliva	133.87 ng/uL	1.87	0.68	7.9	50575	92.55	22.20 ng/uL
C2	TH-Puregene Saliva	114.97 ng/uL	1.79	0.98	8	55861	90.63	13.80 ng/uL
D2	TH-PrepIT-Saliva	400.47 ng/uL	1.8	1.03	6.8	>60000	85.42	92.60 ng/uL

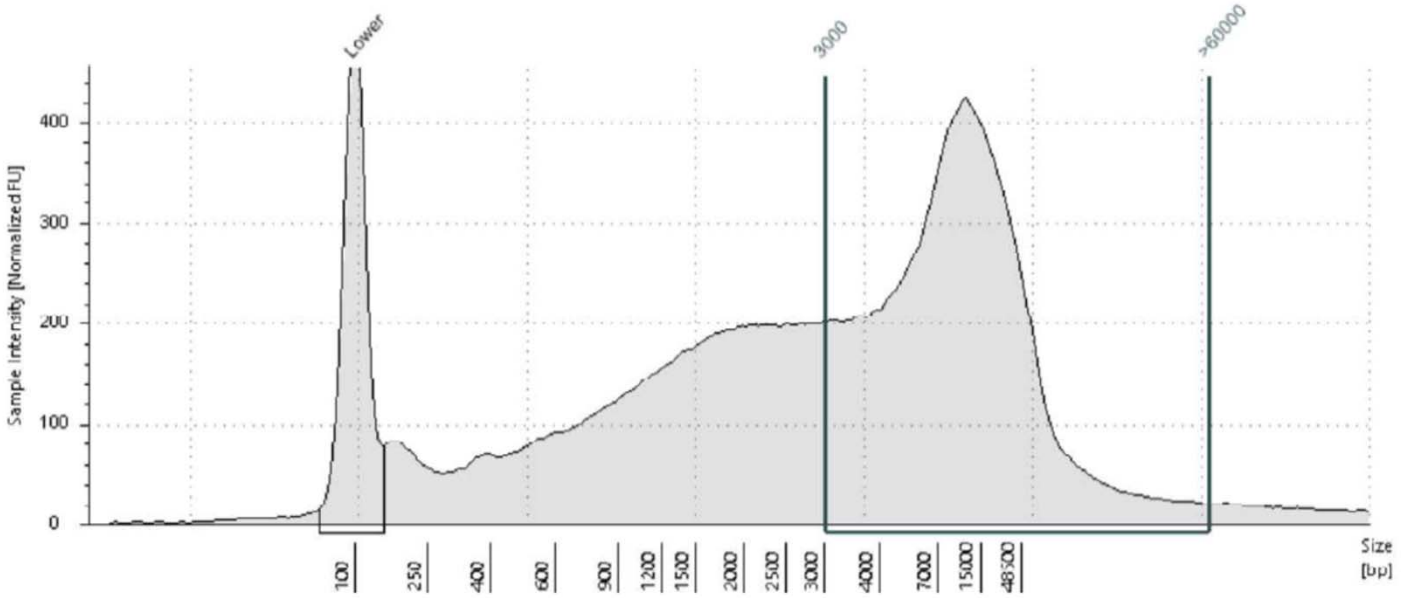
% region (3kb- 60kb): PBMC



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total	Region Comment	Color
3000	>60000	-	63.6	3.14	89.69		

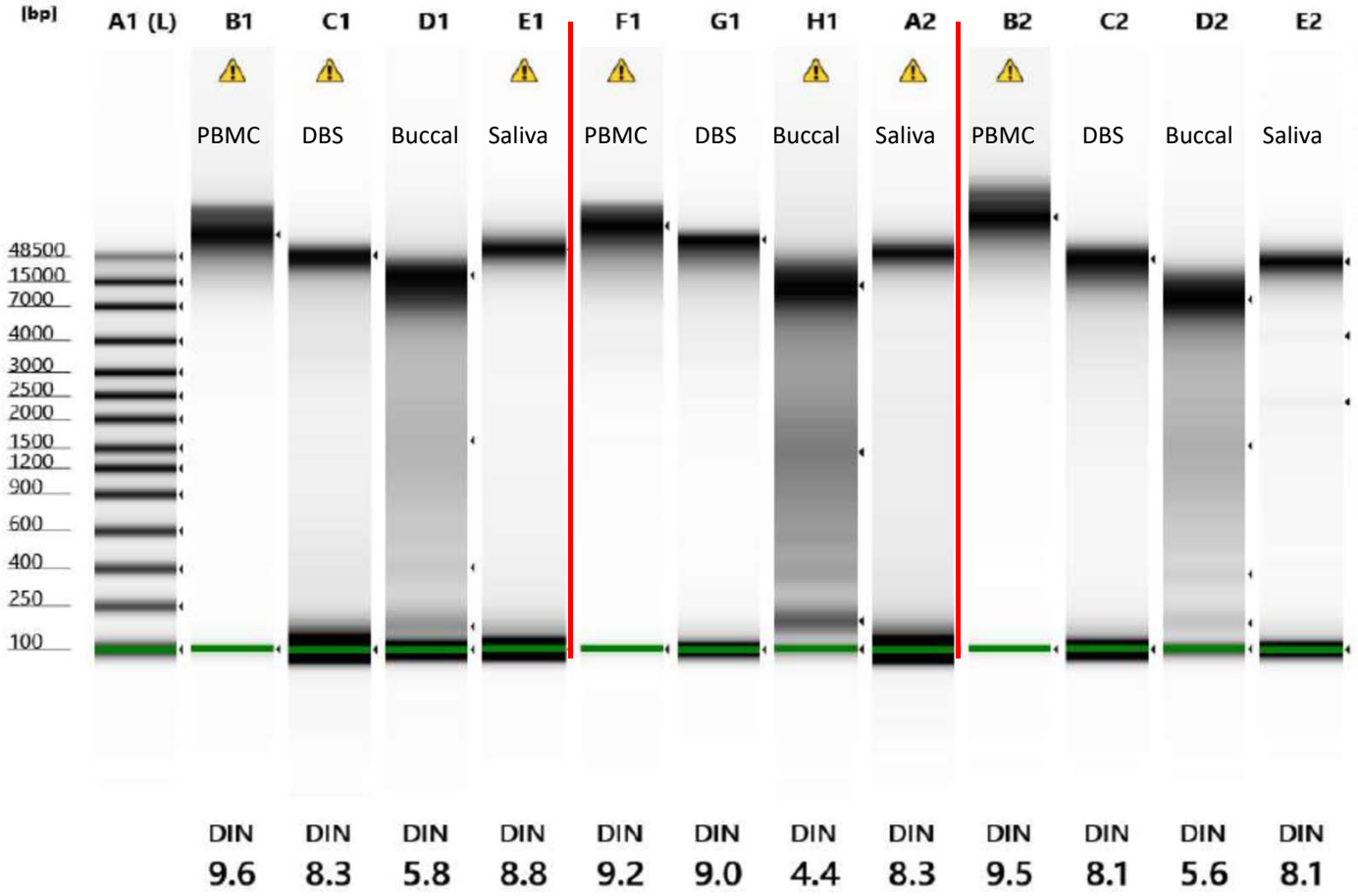
% region (3kb- 60kb): Buccal



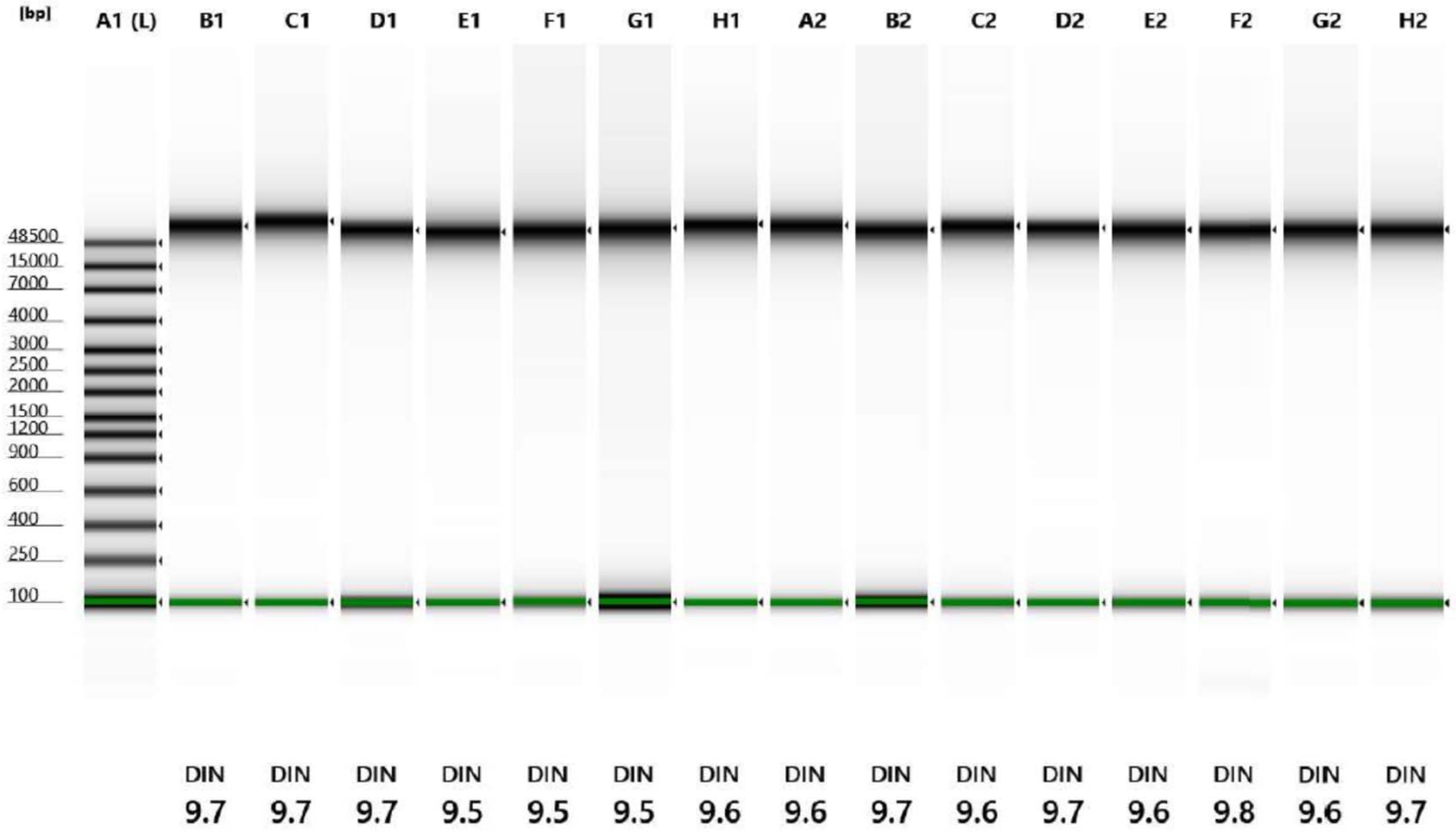
Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	% of Total	Region Comment	Color
3000	>60000	-	41.7	8.21	53.37		

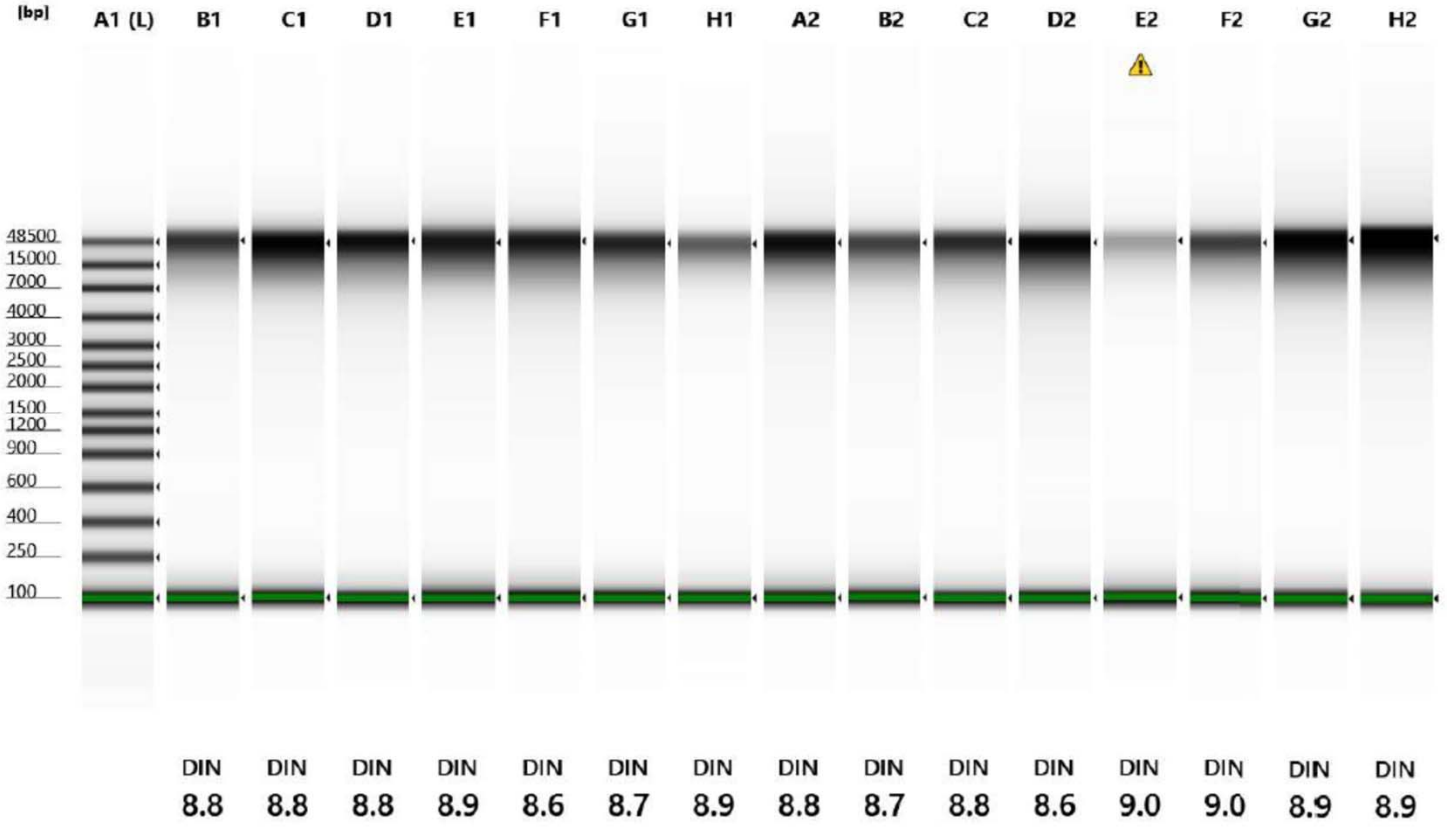
TapeStation bioanalyzer



DNA extraction comparison study (Puregene kit)



DNA extraction comparison study (QIAamp kit)



Descriptive statistics: All tissue cells

Tissue cell	DIN (SD)	% region 3kb-60kb (SD)	260/280 (SD)	260/230 (SD)
PBMC	8.96 (.95)	85% (6.5%)	1.86 (.03)	1.15 (.53)
DBS	8.35 (.80)	75% (16%)	1.69 (.15)	0.84 (.41)
Buccal	5.79 (.99)	53% (11%)	1.80 (.06)	0.75 (.22)
Saliva	8.54 (.58)	73% (11%)	1.92 (.15)	0.73 (.24)

All tissue cells (N=312/132)

	Nanodrop conc.	260/280	260/230	TapeStation DIN	TapeStation conc.
260/280	.077				
260/230	.538**	.121*			
TapeStation DIN	.083	.086	.318*		
TapeStation conc.	.680**	.146*	.627**	.114	
% region 3kb- 60kb	.055	.093	.362**	.873**	.191*

PBMCs (N=79/33)

	Nanodrop conc.	260/280	260/230	TapeStation DIN	TapeStation conc.
260/280	-.292**				
260/230	.593**	-.157			
TapeStation DIN	.413*	.028	.670**		
TapeStation conc.	.559**	-.186	.653**	.419*	
% region 3kb- 60kb	-.241	.149	.147	.495**	.275

DBS (N=79/33)

	Nanodrop conc.	260/280	260/230	TapeStation DIN	TapeStation conc.
260/280	-.212				
260/230	-.470**	.449**			
TapeStation DIN	-.083	.127	.510**		
TapeStation conc.	.086	.261	.602**	.464**	
% region 3kb- 60kb	-.171	.334*	.637*	.853**	.736**

Buccal (N=79/33)

	Nanodrop conc.	260/280	260/230	TapeStation DIN	TapeStation conc.
260/280	.310*				
260/230	.711**	.586**			
TapeStation DIN	.292	.409*	.355*		
TapeStation conc.	.310	.000	.083	.030	
% region 3kb- 60kb	.229	.472**	.327	.931**	.050

Saliva (N=79/33)

	Nanodrop conc.	260/280	260/230	TapeStation DIN	TapeStation conc.
260/280	-.363*				
260/230	.743**	-.221*			
TapeStation DIN	.384*	.052	.457*		
TapeStation conc.	.753**	-.371*	.721**	.387*	
% region 3kb- 60kb	.561**	-.544**	.694**	.413*	.670**

Analytic plan- DNA integrity

- ***Aim 1***: Explore within-person variability in DIN as a function of tissue type.
- ***Aim 2:*** Explore relationships between DIN and other metrics of DNA quality and integrity.
- ***Aim 3***: Explore relationship between DIN and indictors of telomere length assay precision and quality (SD and CV across replicate T and S estimates and SD across replicate Ct values)
- ***Aim 4***: Explore whether DIN moderates the relationship between TL and metrics of external validity (age and tissue type).

Experimental Results (2020), 1–11
doi:10.1017/exp.2020.58

CAMBRIDGE
UNIVERSITY PRESS

BIOMEDICAL SCIENCES

SUPPLEMENTARY-RESULT

NOVEL-RESULT

Uninterruptible Power Supply Improves Precision and External Validity of Telomere Length Measurement *via* qPCR

Waylon J. Hastings[1], Dan T.A. Eisenberg[2] and Idan Shalev[1]

[1]Department of Biobehavioral Health, The Pennsylvania State University, University Park PA, USA and [2]Department of Anthropology, University of Washington, Seattle, WA, USA
Corresponding author. E-mail: whastings2012@gmail.com

EXPERIMENTAL DESIGN

Sample

- DNA extracted from buffy coat (N=94) and buccal epithelial cells (N=269) of participants in the Female Growth and Development Study (PI: Noll; R01-HD052533)
  - Grandmothers (N=26; age 52.6– 72.2)
  - Mothers (N=106; age 29.1 – 43.6)
  - Offspring (N=126, 45.4% male; age 0.5 – 24.9)

qPCR Details

- T/S Ratio per Cawthon (2002)
- 2221 replicate reactions
- 34 qPCR runs (17 T & 17 S)
- Rotor-Gene Q Thermocycler (Qiagen)

Hastings et al., 2020. *Experimental Results.* 1-11. doi: 10.1017/exp.2020.58

EXPERIMENTAL DESIGN

UPS

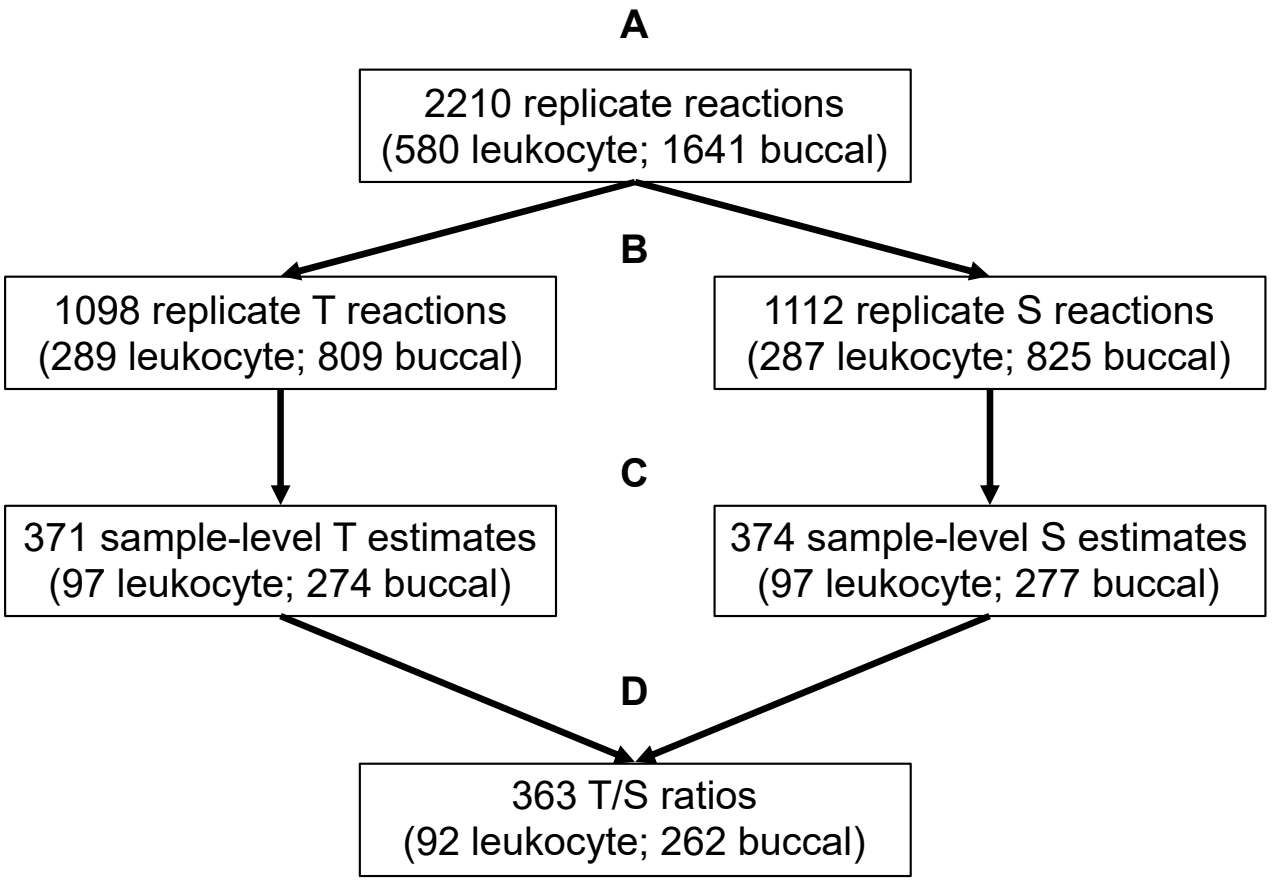
- Back-UPS Pro 700 (APC)
  - Provides backup power, surge protection, and automatic voltage regulation
- Utilized on ~53% of qPCR runs (9 T & 9 S)

Outcomes

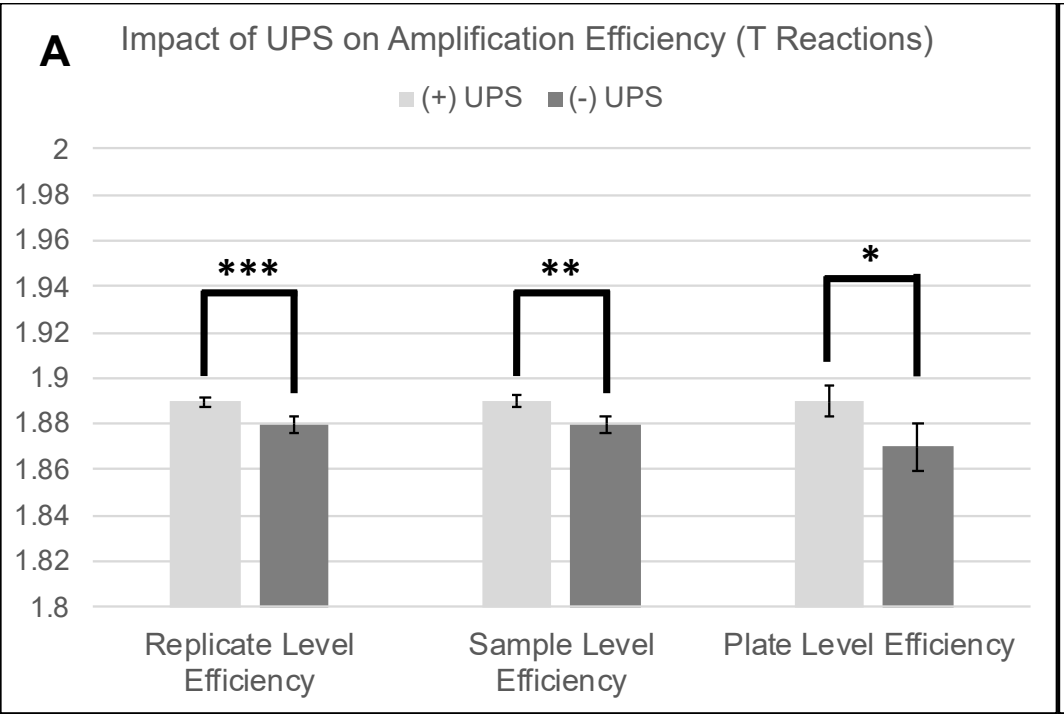
- Amplification Efficiency (via LinRegPCR)
- Standard deviation and CV across technical replicates
- T/S ratio correlations
  - Age
  - Across tissue within-person
  - Parent-offspring

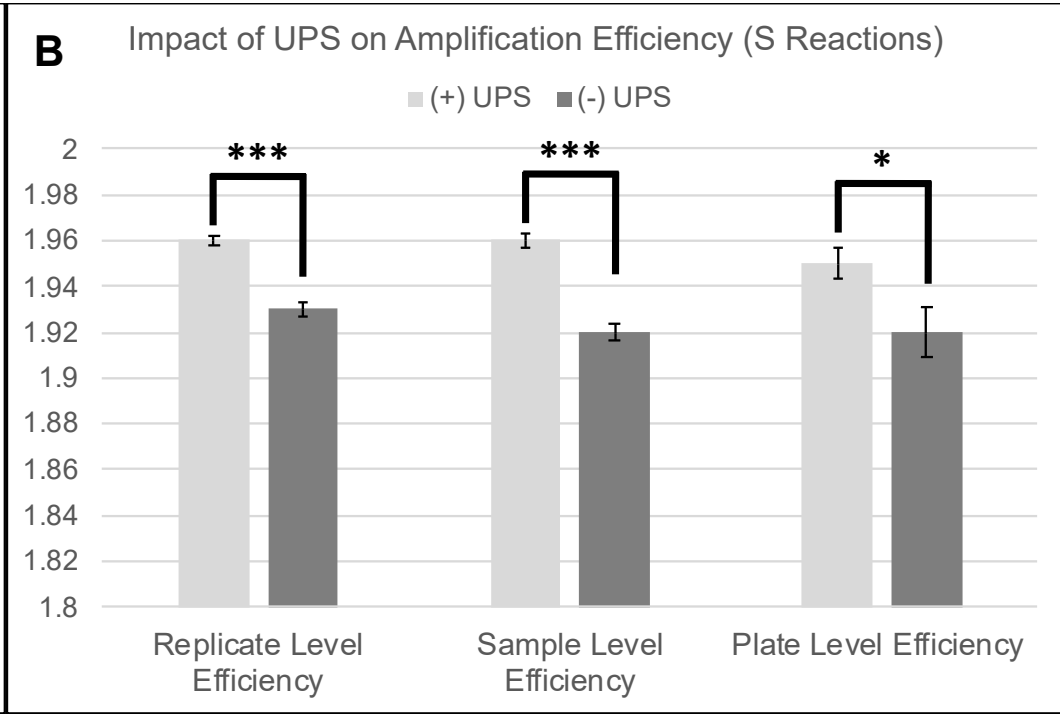
Hastings et al., 2020. *Experimental Results.* 1-11. doi: 10.1017/exp.2020.58

SAMPLE FLOW


A
2210 replicate reactions
(580 leukocyte; 1641 buccal)
B
1098 replicate T reactions
(289 leukocyte; 809 buccal)
1112 replicate S reactions
(287 leukocyte; 825 buccal)
C
371 sample-level T estimates
(97 leukocyte; 274 buccal)
374 sample-level S estimates
(97 leukocyte; 277 buccal)
D
363 T/S ratios
(92 leukocyte; 262 buccal)

UPS RESULTS: EXPONENTIAL AMPLIFICATION

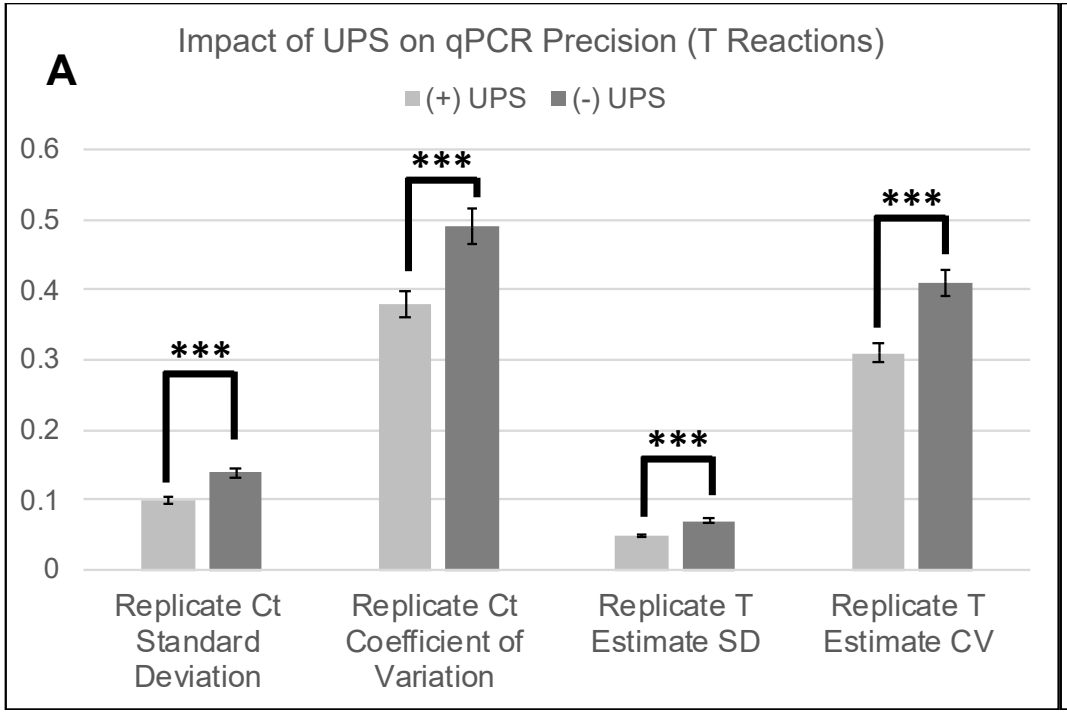


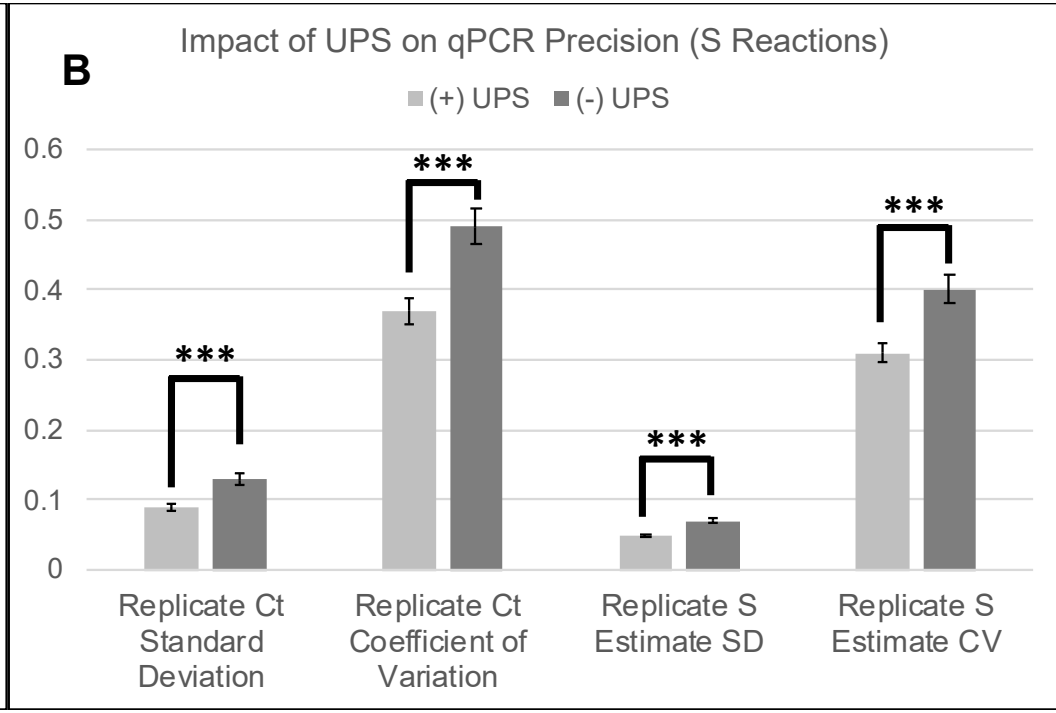


***p<0.001; **p<0.01; *p<0.05

Hastings et al., 2020. *Experimental Results.* 1-11. doi: 10.1017/exp.2020.58

UPS RESULTS: TECHNICAL VARIABILITY





***p<0.001

Hastings et al., 2020. *Experimental Results.* 1-11. doi: 10.1017/exp.2020.58

UPS RESULTS: EXTERNAL VALIDITY

Table 3: Comparing Metrics of External Validity by UPS Status			
Leukocyte-Buccal Correlation of Plate-Level T/S Ratios			
	r (p-value)	**83.4% CI**	**Sample Size Reduction**
(-) UPS	0.62 (<0.001)	[0.47 , 0.74]	61%
(+) UPS	0.92 (<0.001)	[0.88 , 0.95]	
*Correlations controlling for sex and age			
Correlation Between Age and Plate-Level T/S Ratios			
	r (p-value)	**83.4% CI**	**Sample Size Reduction**
(-) UPS	-0.13 (0.085)	[-0.23 , -0.02]	25%
(+) UPS	-0.15 (0.048)	[-0.26 , -0.05]	
*Correlations controlling sex and tissue (leukocyte/buccal)			
Parent-Offspring Correlation of Plate-Level T/S Ratios			
	r (p-value)	**83.4% CI**	**Sample Size Reduction**
(-) UPS	0.74 (<0.001)	[0.65 , 0.80]	8%
(+) UPS	0.78 (<0.001)	[0.70 , 0.84]	
*Correlations controlling for offspring sex, parental age, offspring age, and tissue (leukocyte/buccal)			

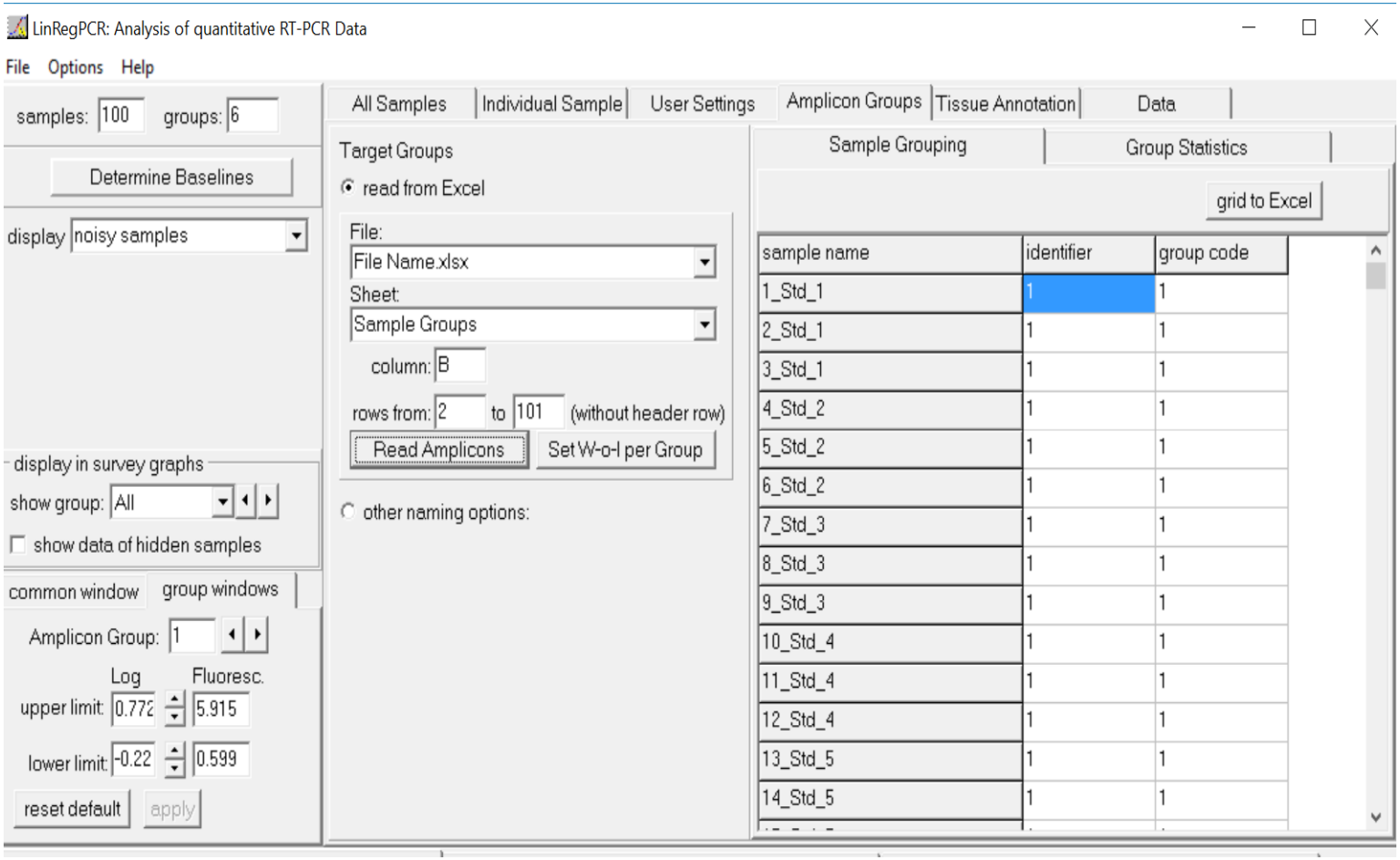
Hastings et al., 2020.

IMPACT OF EFFICIENCY APPROACH ON TL PRECISION AND EXTERNAL VALIDITY

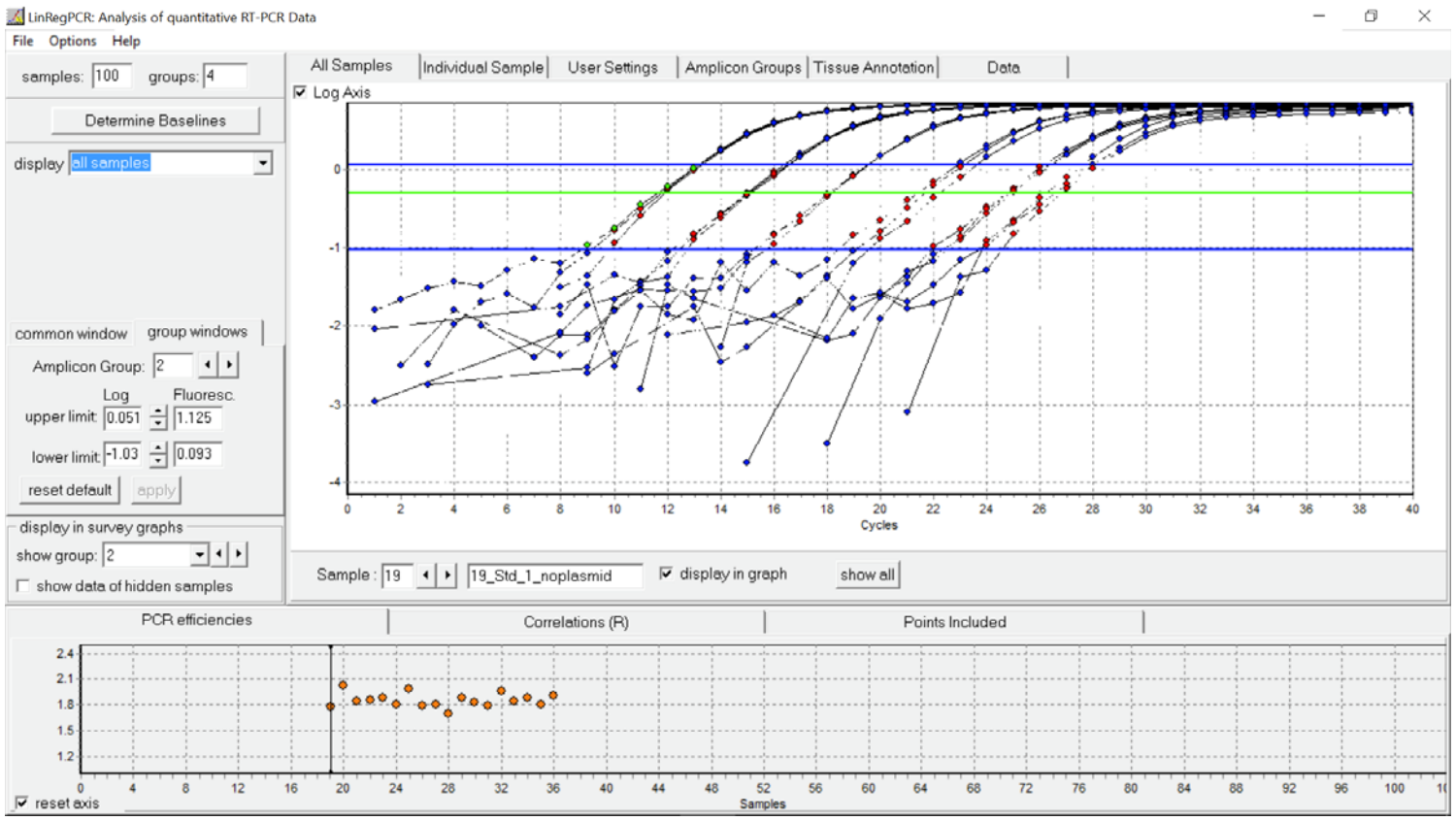
USING LINREGPCR STEP 1: IMPORT RAW FLOURESCENCE DATA

	A	B	C	D	E	F	G	H	I
1	Excel Raw Data Export								
2	Copyright (c) 2010 QIAGEN GmbH. All Rights Reserved.								
3	File	Optimize_Round3_55cycle.rex							
4	Date	####							
5	Time	####							
6									
7									
8	Channel Cycling A.Green								
9									
10	ID	Page	1	2	3	4	5	6	
11	Background		0	0	0	0	0	0	
12	1	Std 1_	0.9990845	1.0296318	1.0431067	1.0466515	1.0104524	1.0071972	1.04676
13	2	Std 1_	0.9422446	0.9502011	0.9367005	0.9491112	0.9351746	0.9478396	0.97291
14	3	Std 1_	0.9857328	1.0223545	1.0427362	1.0178858	1.0250793	1.0131991	1.04371
15	4	Std 2_	1.0437171	0.9906375	0.996821	0.9881743	0.9930353	1.0104524	0.99448
16	5	Std 2_	0.9760688	1.0067903	0.976482	0.9962968	0.9911825	0.9839889	0.95244
17	6	Std 2_	0.9282572	0.9352509	0.948806	0.9393689	0.9423972	0.9302135	0.90867
18	7	Std 3_	1.0334173	0.9891915	1.0315099	1.0100383	0.9856238	0.9863197	0.95271
19	8	Std 3_	0.9727626	0.9788154	0.975521	0.9558017	0.94234	0.9464408	0.91815
20	9	Std 3_	1.0254063	1.0241151	1.0399023	1.0416462	1.0323315	0.9947792	1.01375
21	10	Std 4	1.1074235	1.1034307	1.0977595	1.0804659	1.0926847	1.0789082	1.08044

USING LINREGPCR STEP 2: DEFINING AMPLICONS



USING LINREGPCR STEP 3: EVALUATING PROFILES BY AMPLICON GROUP

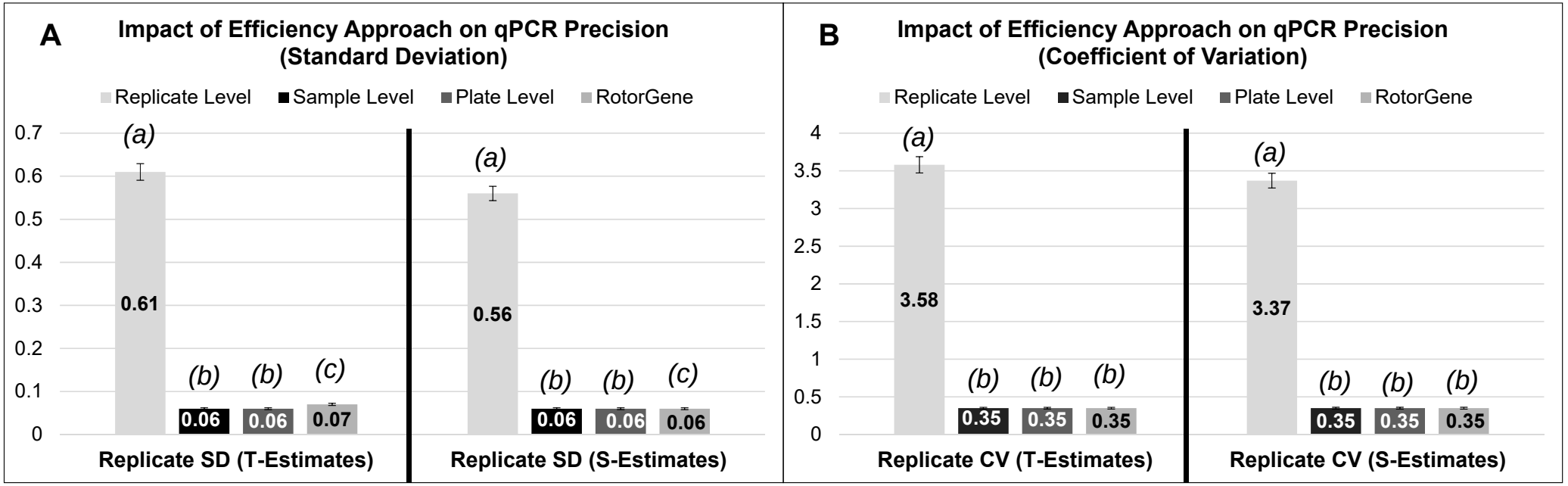


USING LINREGPCR STEP 4: EXPORT RESULTS

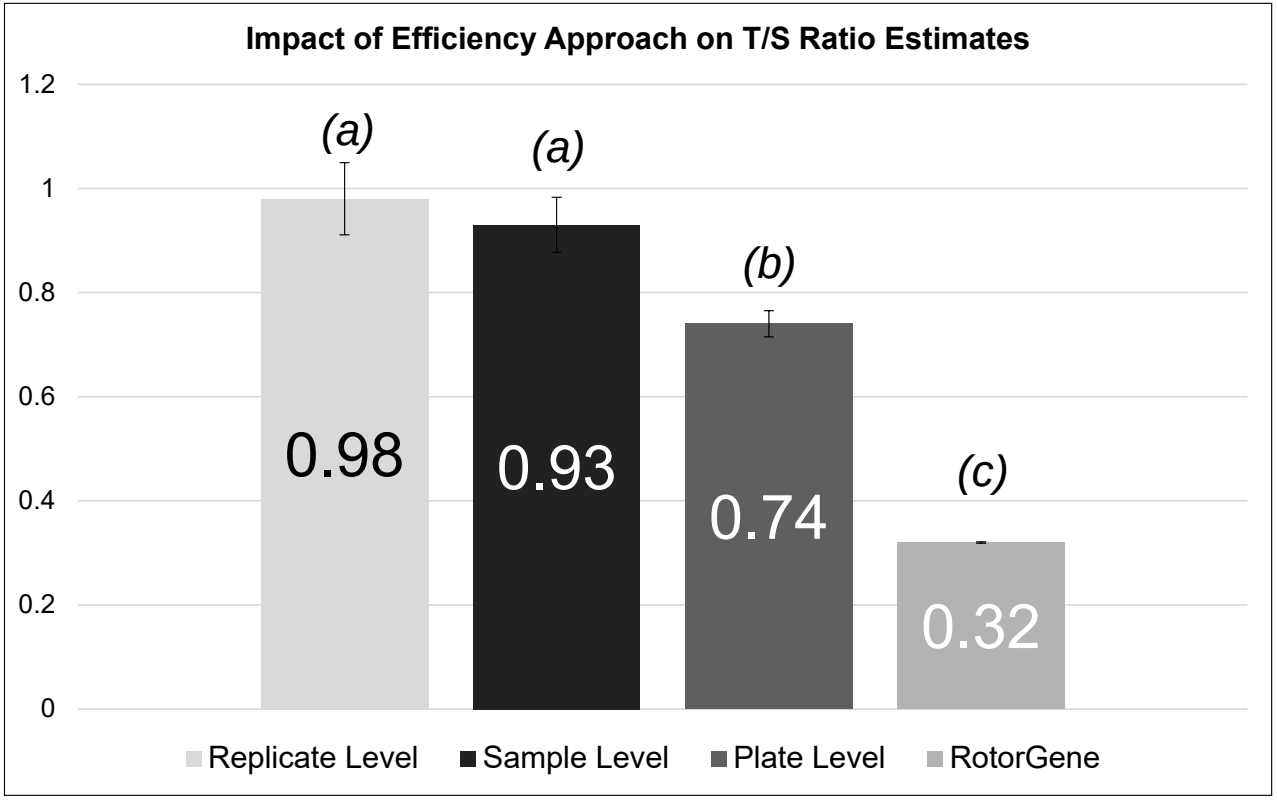
	A	B	C	D	E	F	G	H	I	J
1	Analysis of Real Time PCR data	version:2020.0	WoL: amplicon group			Chemistry: DNA binding dyes	N0 = threshold /(Eff_mean^Cq)			LEGEND
2	analysis date:10/15/2020		points in WoL: 4			Input: ds-DNA				
3	Input Sheet: RotorGene Export		Threshold: common							Sample Use:
4	name	indiv_PCR_eff	Amplicon	threshold	mean_PCR_eff	Cq	N0	Sample_Use	Quality_checks	1: used for W-o-L setting
5	Std 1	2.165	2	0.400	1.817	10.686	6.78E-04	1 0 3	- - - - - 5 6 - - - - - - -	2: contributes to mean PCR efficiency
6	Std 1	1.815	2	0.400	1.817	10.233	8.89E-04	1 2 3	0 - - - - - - - - - - - - -	3: N0 value calculated
7	Std 1	1.831	2	0.400	1.817	10.468	7.72E-04	0 2 3	0 - - - - - - - - - - - - -	0: not used / calculated
8	Std 2	1.763	2	0.400	1.817	14.067	9.01E-05	1 2 3	0 - - - - - - - - - - - - -	
9	Std 2	1.973	2	0.400	1.817	14.463	7.11E-05	1 0 3	- - - - - 5 6 - - - - - - -	Quality Checks:
10	Std 2	1.846	2	0.400	1.817	14.277	7.95E-05	1 2 3	0 - - - - - - - - - - - - -	0: passed all checks
11	Std 3	1.000	2	0.400	1.817	0.000	-9.99E+02	0 0 0	- 1 - 3 - 5 6 - - - - - -	1: no amplification
12	Std 3	1.986	2	0.400	1.817	17.946	8.89E-06	1 0 3	- - - - - 5 6 - - - - - - -	2: baseline error
13	Std 3	1.831	2	0.400	1.817	17.689	1.04E-05	1 2 3	0 - - - - - - - - - - - - -	3: no plateau
14	Std 4	1.846	2	0.400	1.817	20.690	1.73E-06	1 2 3	0 - - - - - - - - - - - - -	4: noisy sample
15	Std 4	2.066	2	0.400	1.817	21.265	1.23E-06	1 0 3	- - - - - 5 6 - - - - - - -	5: PCR efficiency outside 10%
16	Std 4	1.803	2	0.400	1.817	21.103	1.35E-06	0 2 3	0 - - - - - - - - - - - - -	6: excluded from mean Eff
17	Std 5	1.741	2	0.400	1.817	23.685	2.89E-07	1 2 3	0 - - - - - - - - - - - - -	7: excluded by user
18	Std 5	2.034	2	0.400	1.817	24.206	2.12E-07	1 0 3	- - - - - 5 6 - - - - - - -	8: included by user
19	Std 5	1.878	2	0.400	1.817	24.034	2.35E-07	1 2 3	0 - - - - - - - - - - - - -	9: manual baseline
20	Std 6	2.007	2	0.400	1.817	25.804	8.16E-08	1 0 3	- - - - - 5 6 - - - - - - -	
21	Std 6	1.709	2	0.400	1.817	25.442	1.01E-07	0 0 3	- - - - - 5 6 - - - - - - -	if amplicon groups are defined the rules are applied per group
22	Std 6	1.871	2	0.400	1.817	25.844	7.97E-08	1 2 3	0 - - - - - - - - - - - - -	
23	C1_TELO	1.890	1	0.400	1.801	14.136	9.73E-05	0 2 3	0 - - - - - - - - - - - - -	
24	C1_TELO	2.119	1	0.400	1.801	14.128	9.78E-05	1 0 3	- - - - - 5 6 - - - - - - -	
25	C1_TELO	1.771	1	0.400	1.801	13.952	1.08E-04	0 2 3	0 - - - - - - - - - - - - -	
26	C2_TELO	2.112	1	0.400	1.801	14.636	7.26E-05	1 0 3	- - - - - 5 6 - - - - - - -	
27	C2_TELO	1.816	1	0.400	1.801	14.083	1.00E-04	1 2 3	0 - - - - - - - - - - - - -	
28	C2_TELO	1.858	1	0.400	1.801	13.886	1.13E-04	1 2 3	0 - - - - - - - - - - - - -	
29	C3_TELO	1.955	1	0.400	1.801	13.151	1.74E-04	0 0 3	- - - - - 5 6 - - - - - - -	
30	C3_TELO	1.738	1	0.400	1.801	12.841	2.09E-04	1 2 3	0 - - - - - - - - - - - - -	
31	C3_TELO	1.000	1	0.400	1.801	0.000	-9.99E+02	0 0 0	- - 2 3 - 5 6 - - - - - -	
32	C1_SCG	2.037	2	0.400	1.817	24.538	1.74E-07	1 0 3	- - - - - 5 6 - - - - - - -	
33	C1_SCG	2.013	2	0.400	1.817	24.392	1.90E-07	1 0 3	- - - - - 5 6 - - - - - - -	
34	C1_SCG	1.990	2	0.400	1.817	24.480	1.80E-07	1 0 3	- - - - - 5 6 - - - - - - -	
35	C2_SCG	2.106	2	0.400	1.817	24.388	1.90E-07	1 0 3	- - - - - 5 6 - - - - - - -	
36	C2_SCG	1.794	2	0.400	1.817	23.862	2.60E-07	1 2 3	0 - - - - - - - - - - - - -	
37	C2_SCG	2.066	2	0.400	1.817	24.471	1.81E-07	0 0 3	- - - - - 5 6 - - - - - - -	
38	C3_SCG	1.763	2	0.400	1.817	22.700	5.20E-07	1 2 3	0 - - - - - - - - - - - - -	

RotorGene Export | Sample Groups | Plate1_IFNB1_LinRegExport | Plate1_TELO_LinRegExport | Conversion Factors | Plate1aTL | +

EFFICIENCY RESULTS: PRECISION



EFFICIENCY RESULTS: T/S RATIO ESTIMATES



EFFICIENCY RESULTS: EXTERNAL VALIDITY

Table 4: Comparing Metrics of External Validity by Efficiency Approach

Leukocyte-Buccal Correlation of T/S Ratios		
	r (p-value)	**83.4% CI**
Replicate Efficiency T/S	0.25 (0.025)	[0.10 , 0.39]
Sample Effiiciency T/S	0.41 (<0.001)	[0.27 , 0.53]
Plate Efficiency T/S	0.83 (<0.001)	[0.77 , 0.87]
RotorGene Efficiency T/S	0.73 (<0.001)	[0.65 , 0.79]
*Correlations controlling for sex and age		
Correlation Between Age and T/S Ratios		
	r (p-value)	**83.4% CI**
Replicate Efficiency T/S	-0.04 (0.411)	[-0.12 , 0.03]
Sample Effiiciency T/S	-0.09 (0.084)	[-0.16 , -0.02]
Plate Efficiency T/S	-0.14 (0.009)	[-0.21 , -0.07]
RotorGene Efficiency T/S	-0.06 (0.230)	[-0.14 , 0.01]
*Correlations controlling for sex and tissue (leukocyte/buccal)		
Parent-Offspring Correlation of T/S Ratios		
	r (p-value)	**83.4% CI**
Replicate Efficiency T/S	0.30 (<0.001)	[0.20 , 0.40]
Sample Effiiciency T/S	0.35 (<0.001)	[0.24 , 0.44]
Plate Efficiency T/S	0.79 (<0.001)	[0.75 , 0.83]
RotorGene Efficiency T/S	0.78 (<0.001)	[0.73 , 0.82]
*Correlations controlling for offspring sex, parental age, offspring age, and tissue (leukocyte/buccal)		

$$T/S = \frac{Efficiency_T{}^{Ct}}{Efficiency_S{}^{Ct}}$$

	LinRegPCR		**RotorGene**	
Target	Telo	SCG	Telo	SCG
Efficiency (SD)	1.8794 (0.026)	1.9367 (0.030)	1.9923 (0.004)	1.9924 (0.006)