How telomeres protect chromosome ends from engaging the DNA damage response

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NIA
January 18, 2011
Mammalian telomeres

Telomeres

telomere uncapping

telomere dysfunction

absence of telomerase

DNA damage response

wt p53 p53 loss

apoptosis senescence cancer initiation

Deng et al., Nat Rev Cancer 2008
Consequence of natural telomere attrition: multiple non-reciprocal chromosomal translocations in tumors with short telomeres

Breast tumor TD 574

Artandi et al., Nature 2000
Massive end-to-end chromosome fusions in the absence of Trf2
Dysfunctional telomeres promote genomic instability

Epithelial Renewal And Advancing Age

Critical Telomere Attrition
Telomere Dysfunction

Anaphase Bridge

DSB

p53

BFB Cycles

Cytokinesis

Aneuploidy
NRTs
Amplifications
Deletions

Telomerase Reactivation

Invasive carcinoma

Carcinoma in situ
Six subunits in shelterin interacts with the t-loop: TRF1, TRF2, TIN2, RAP1, TPP1 and POT1

POT1 - Protection of Telomere 1

Required for telomere end protection

Negative regulator of telomere length
### POT1 structure

<table>
<thead>
<tr>
<th>POT1 Type</th>
<th>OB1</th>
<th>OB2</th>
<th>TPP1 BD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse Pot1a</strong></td>
<td><img src="image1" alt="Diagram" /></td>
<td><img src="image2" alt="Diagram" /></td>
<td><img src="image3" alt="Diagram" /></td>
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<tr>
<td><strong>Mouse Pot1b</strong></td>
<td><img src="image4" alt="Diagram" /></td>
<td><img src="image5" alt="Diagram" /></td>
<td><img src="image6" alt="Diagram" /></td>
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<tr>
<td><strong>Human POT1</strong></td>
<td><img src="image7" alt="Diagram" /></td>
<td><img src="image8" alt="Diagram" /></td>
<td><img src="image9" alt="Diagram" /></td>
</tr>
</tbody>
</table>

Lei et al., *Nature Struct and Mol Biol* 2004
Conditional Deletion of Mouse \textit{Pot1a}

A  

\begin{center}
\begin{tikzpicture}
  \node (targeting) at (0,0) {
    \begin{minipage}[t]{0.8\textwidth}
      \includegraphics[width=\textwidth]{targeting_vector_diagram.png}
    \end{minipage}
  };

  \node (targeted) at (0,-3) {
    \begin{minipage}[t]{0.8\textwidth}
      \includegraphics[width=\textwidth]{targeted_allele_diagram.png}
    \end{minipage}
  };

  \node (null) at (0,-6) {
    \begin{minipage}[t]{0.8\textwidth}
      \includegraphics[width=\textwidth]{null_allele_diagram.png}
    \end{minipage}
  };

  \draw[->] (targeting) -- (targeted);
  \draw[->] (targeted) -- (null);

  \node (DT) at (-2,0.5) {DT};
  \node (B) at (-1,0.5) {B};
  \node (S) at (1,0.5) {S};

  \node (loxp) at (0.2,0.5) {loxP};

  \node (frt) at (0.75,0.5) {frt};
  \node (ATG) at (1.25,0.5) {ATG};

  \node (neo) at (0.5,-0.3) {neo};

  \node (4) at (0.75,-0.3) {4};

  \node (7k) at (0.75,-2) {7 \text{ kb}};

  \node (11.8k) at (1.25,-2) {11.8 \text{ kb}};

  \node (NotI) at (2.25,0.5) {NotI};

  \node (targeting_vector) at (4,0) {targeting vector};

  \node (targeted_allele) at (4,-3) {targeted allele \ Pot1^{\text{rec}}};

  \node (null_allele) at (4,-6) {null allele \ Pot1^{\Delta}};
\end{tikzpicture}
\end{center}

B  

\begin{center}
\begin{tabular}{ccc}
\textbf{Pot1}^{++} & \textbf{Pot1}^{\text{rec}\Delta} & \textbf{Pot1}^{\Delta\Delta} \\
\includegraphics[width=0.3\textwidth]{mPot1a_blot.png} & \includegraphics[width=0.3\textwidth]{mPot1a_blot_rec.png} & \includegraphics[width=0.3\textwidth]{mPot1a_blot_dd.png} \\
\textbf{control} & \textbf{control} & \textbf{control} \\
\end{tabular}
\end{center}

C  

\begin{center}
\begin{tabular}{ccc}
\textbf{Pot1}^{\text{rec}\Delta} & \textbf{Pot1}^{\Delta\Delta} \\
\includegraphics[width=0.3\textwidth]{mPot1a_blot_rec.png} & \includegraphics[width=0.3\textwidth]{mPot1a_blot_dd.png} \\
\textbf{GAPDH} & \textbf{GAPDH} & \textbf{GAPDH} \\
\end{tabular}
\end{center}

D  

\begin{center}
\begin{tabular}{ccc}
\textbf{Pot1a-myc} & \textbf{Pot1a}^{++} & \textbf{Pot1}^{\text{rec}\Delta} & \textbf{Pot1}^{\Delta\Delta} \\
\includegraphics[width=0.3\textwidth]{Pot1a_myc_blot.png} & \includegraphics[width=0.3\textwidth]{Pot1a_myc_blot++.png} & \includegraphics[width=0.3\textwidth]{Pot1a_myc_blot_rec.png} & \includegraphics[width=0.3\textwidth]{Pot1a_myc_blot_dd.png} \\
\textbf{*} & \textbf{*} & \textbf{*} & \textbf{*} \\
\end{tabular}
\end{center}
Elevated DNA damage response in *Pot1a* deleted cells

**A**

10 Gy γ-IR, Pot1<sup>rec/Δ</sup> Pot1<sup>Δ/+</sup> Pot1<sup>rec/Δ</sup> Pot1<sup>Δ/Δ</sup>

γH2AX

**B**

53BP1 telomere merge magnified

Pot1<sup>Δ/Δ</sup>, p53<sup>+/−</sup>

**C**

<table>
<thead>
<tr>
<th>Pot1&lt;sup&gt;rec/Δ&lt;/sup&gt;</th>
<th>Pot1&lt;sup&gt;Δ/+&lt;/sup&gt;</th>
<th>Pot1&lt;sup&gt;rec/Δ&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-IR - +</td>
<td>AdCre - +</td>
<td>AdCre - +</td>
</tr>
</tbody>
</table>

- ATM-Ser1981-P
- Chk2-P
- Chk2
- γ-H2AX-P
- γ-tubulin
Pot1a loss leads to multiple cytogenetic aberrations and malignant transformation

A

B

C

D

Pot1aΔΔ, p53−/− sarcoma

Pot1aΔΔ, p53−/−
Acceleration of MMTV-Cre\(^+\); Pot1a\(^{FF}\) breast tumors in the absence of p53

**Graphs:**

- % tumor free survival over months for different genotypes:
  - Cre\(^-\) Pot1a\(^{FF}\) n=21
  - Cre\(^+\) Pot1a\(^{FF}\) n=23
  - Cre\(^+\) Pot1a\(^{FF}\) p53\(^{FF}\) n=22
  - Cre\(^+\) Pot1a\(^{FF}\) p53\(^{F/+}\) n=31

**Images:**

- Image of mouse tail and tumor

**Legend:**

- Pot1a\(^{F/F}\) → tail
- Pot1a\(^{Δ/Δ}\) → tumor

Ling Wu
Massive telomere amplifications in MMTV-Cre$^+$ Pot1a$^{Δ/Δ}$ breast tumors
POT1a is required for telomere end protection

DNA damage response

C-strand resection/ G-overhang elongation

Overhang removal NHEJ

T-loop

TRF2

Pot1Δ/Δ

Homologous recombination

Telomere double minutes/circles

Pot1Δ/Δ

Genomic instability

TIFs

p53

tumorigenesis
Conditional deletion of mouse *Pot1b*

**A**

- Targeting vector
- wt allele
- Recombined allele: Pot1b<sup>rec</sup>
- Null allele: Pot1b<sup>-/-</sup>

**B**

- Targeted ES cell
- wt ES cell
- wt: 11Kb
- mutant: 7.8Kb
- loading control

**C**

- Pot1b<sup>+/−</sup>
- Pot1b<sup>−/−</sup> #1
- Pot1b<sup>−/−</sup> #2
- Pot1b transcript
- GPDH

**D**

- Mice images
Generating Pot1b null alleles

5'-TTAGGGTTAGGGTTAGGGTTAGGG
3'-G(TTAGGG)n-3'

3'-AAGCCCAAGCCCAAGCCCAAGCCC

POT1b
TRF1 TRF2/Rap1 telomerase
Deletion of Pot1b in the setting of telomerase deficiency results in diminished lifespan
Complete bone marrow failure in Pot1b^-/- mTR^+/- mice

A

WT  Pot1b^-/- mTR^+/-  Pot1b^-/- mTR^+/-  Pot1b^-/- mTR^-/

B

CD11b  Gr-1

WT  Pot1b^-/- mTR^+/-
Depletion of hematopoietic progenitor/stem cells in Pot1b\(^{-/-}\) mTR\(^{+/-}\) bone marrow

WT (6 months) 2 months 4 months 6 months

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<th>10^2</th>
<th>10^1</th>
<th>10^0</th>
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<tr>
<td>C-KIT</td>
<td>2.04%</td>
<td>1.34%</td>
<td>0.39%</td>
<td>&lt;0.01%</td>
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<tr>
<td>Sca-1</td>
<td>0.23%</td>
<td>0.13%</td>
<td>0.06%</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Lin-</td>
<td></td>
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</tbody>
</table>

Eric Wang
Peripheral blood defects in Pot1b−/− mTR+/− mice

(A) Bar graphs showing hematological parameters:
- RBC (Red Blood Cells) $\times 10^6/\mu L$:
  - WT: 15.2% ± 3.2%
  - Pot1b−/− mTR+/-: 5.0% ± 1.2%
- Platelets $\times 10^9/\mu L$:
  - WT: 12.6% ± 0.1%
  - Pot1b−/− mTR+/-: 7.5% ± 2.5%
- WBC (White Blood Cells) $\times 10^3/\mu L$:
  - WT: 3.2% ± 8.8%
  - Pot1b−/− mTR+/-: 16.4% ± 8.8%

(B) Light microscopy images:
- a) WT
- b) Pot1b−/− mTR+/-
- c) Annexin V
- d) 7-AAD

(C) Dot plots showing Annexin V and 7-AAD stains:
- WT: 3.2% Annexin V−/−7-AAD−
- Pot1b−/− mTR+/-: 12.6% Annexin V−/−7-AAD−
Pot1b^{-/-} mTR ^{+/−} mice displays phenotypes resembling Dyskeratosis congenita (DC)

- abnormal skin pigmentation
- nail dystrophy
- BM failure
Dyskeratosis congenita

Clinical characteristics of dyskeratosis congenita

Source: Lichtman MA, Shafer RS, Felgar RE, Wang N:
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Dyskeratosis congenita is a telomere disease

Dyskerin is a pseudouridine synthase; modifies rRNA, spliceosomal RNA, using a H/ACA small guide RNAs

• Mutated in X-linked dyskeratosis congenita (Collins)
  • aplastic anemia
  • pulmonary fibrosis, hyperpigmentation, oral leukoplakia, nail dystrophy
  • very short telomeres

• TERC or TERT are mutated in autosomal dominant DC (Dokal, Greider)

• NOP10, a small ribonucleoprotein, is mutated in autosomal recessive DC (Dokal)

• Heterozygous mutation in TINF2 recently observed in DC patients (Savage, Dokal)
Increased G-strand overhang and progressive telomere shortening in the absence of Pot1b

A

Pot1b\(^{-/-}\) #1

(CCCTAA)\(_4\)

Overhang

(TTAGGG)\(_4\)

Total telomere

WT

Pot1b\(^{-/-}\) #2

WT

B

Pot1b\(^{-/-}\), mTR\(^{++}\)

passage

35 42 1

35 10

35 41

Pot1b\(^{-/-}\), mTR\(^{++}\)

Pot1b\(^{-/-}\), mTR\(^{++}\)

Pot1b\(^{-/-}\), mTR\(^{++}\)

MEFs
Increased chromosome fusion and telomere shortening in Pot1b⁻/⁻ mTR⁺/⁻ cells

A

Mouse Embryonic fibroblasts, passage 30

Pot1b⁻/⁻ mTR⁺/⁺

Pot1b⁻/⁻ mTR⁺⁺

B

mean=3783 TFU
n=1720

frequency

0 20 40 60 80 100 120 140

TFU (x 100)

C

chromosome fusions per metaphase

0 1 2

D

Pot1b⁻/⁻ mTR⁺⁺

mean=4107 TFU
n=2700

frequency

0 20 40 60 80 100 120 140

TFU (x 100)

E

mean=2173 TFU
n=1253

frequency

0 20 40 60 80 100 120 140

TFU (x 100)

D

chromosome fusions per metaphase

0.00 0.25 0.50 0.75 1.00

splenocytes BM
Pot1b<sup>-/-</sup> mTR<sup>+/−</sup> MEFs initiate a DNA damage response at telomeres
Pot1b is required to modulate ss overhang formation

Adapted from Ramiro and Karlseder, Nature 2007

Increased overhang generation
Accelerated telomere shortening
Increased DDR at telomeres
p53-dependent apoptosis
Role of repressor activator protein 1 (RAP1) in telomere end protection

Removal of TRF2 from telomeres results in DDR at telomeres and massive chromosome end fusions.

Removal of TRF2 results in rapid disappearance of Rap1 from telomeres.

In yeast, Rap1 is involved in protecting telomeres from NHEJ independent from TRF2-like homologs.

What are the roles for mammalian Rap1 in telomere end protection?
Knockdown of Rap1 by shRNA does not work well

Knockdown of RAP1 transcripts using 5 shRAP1 constructs.

O’Connor, JBC 2004
Strategy to study the *in vivo* function of Rap1

Replacement of endogenous TRF2 with Rap1 binding deficient mutant
shRNA mediated knockdown of TRF2 results in massive telomere fusions
Characterization of the TRF2-Rap1 interaction

Isothermal titration Calorimetry

$K_d = 16.5$ nM

Ming Lei, U of Michigan
Purification and crystallization of the TRF2\textsubscript{RBM} and Rap1\textsubscript{RCT} complex

Crystal of the TRF2\textsubscript{RBM}-Rap1\textsubscript{RCT} complex
Crystal Structure of the TRF2\textsubscript{RBM}-Rap1\textsubscript{RCT} Complex at 1.95 Å resolution
The TRF2<sub>RBM</sub>-Rap1<sub>RCT</sub> interface

TRF2: L288

Rap1: I318, F336
ITC analysis of the TRF2\textsubscript{RBM}-Rap1\textsubscript{RCT} interface

<table>
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<tr>
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<th>TRF2</th>
<th>RAP1</th>
<th>$K_d$ (nM)</th>
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<td>RAP1\textsubscript{RCT}</td>
<td>16.5</td>
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<tr>
<td>TRF2</td>
<td>RAP1\textsubscript{RCT}</td>
<td>23.9</td>
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<tr>
<td>TRF2\textsubscript{RBM}</td>
<td>RAP1\textsubscript{RCT}\textsubscript{I318R}</td>
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<tr>
<td>TRF2\textsubscript{RBM}</td>
<td>RAP1\textsubscript{RCT}\textsubscript{F336R}</td>
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<tr>
<td>TRF2\textsubscript{RBM} \textsubscript{L288R}</td>
<td>RAP1\textsubscript{RCT}</td>
<td>nd</td>
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A single point mutation inside the hydrophobic pocket is sufficient to disrupt the ability of TRF2 to bind Rap1.
Co-IP analysis the TRF2-Rap1 Interface in mammalian cells

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<th>Rap1</th>
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<td>L318R</td>
<td>F336R</td>
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<td>Myc: Vector</td>
<td>In</td>
<td>IP</td>
<td>In</td>
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<tr>
<td>TRF2</td>
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<td>TRF2</td>
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<td>TRF2 L288R</td>
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<td>IP</td>
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</tbody>
</table>
TRF2 is essential for Rap1 localization to telomeres
The TRF2-Rap1 interaction not required for TRF2 telomere localization
Minimal DNA damage response at telomeres in the absence of Rap1

TRF2, not Rap1, is required to repress the DDR at telomeres.
Rap1 is required to repress telomere HR

CCCTAA  TTAGGG  merge

Table: T-SCE/ chromosome end

Bar chart: Comparison of T-SCE/ chromosome end between TRF2 FL and shTRF2 L286R conditions.
Distinct roles for Rap1 and TRF2 in telomere end protection

Rap1 is not required to repress DDR and NHEJ at telomeres. Rap1 repress HR at telomeres.
Repair of dysfunctional telomeres by the NHEJ pathway

O'Sullivan and Karlseder, Nature Reviews MCB 2010
Trf2 and Pot1a repress distinct DNA damage signaling pathways

Possibility that TRF2 and POT1-TPP1 repress distinct DNA repair pathways at telomeres.

Denchi and de Lange, Nature 2007
Guo et al; EMBO J 2007
Disruption of TRF2 leads to ATM-dependent telomere fusions through C-NHEJ

TIN2
TPP1

5’-TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG(TTAGGG)n-3’
3’-AAGCCCAAGCCCAAGCCCAAGCCC

TRF1

POT1

MRN

53BP1
Ku70/80
DNA-PK
Ligase 4

C-NHEJ factors

end-to-end chromosome fusions

Dench and de Lange, Nature 2007
Guo et al; EMBO J 2007
Deng et al., Nature 2009
Dimitrova et al., MCB 2009
Disruption of Pot1a-Tpp1 leads to ATR-dependent telomere fusions

Denchi and de Lange, Nature 2007
Guo et al; EMBO J 2007
Disruption of Pot1a-Tpp1 leads to chromosome fusions independent of C-NHEJ.
Chromosome fusions in the absence of Tpp1-Pot1a does not require 53BP1
Chromosome fusions in the absence of Tpp1-Pot1a does not require Lig4
Chromosome fusions in the absence of Tpp1-Pot1a does not require Ku70
A-NHEJ mediated repair of DNA double strand breaks

Rass et al., NSMB 2009
Disruption of Pot1a-Tpp1 leads to alternative NHEJ mediated chromosome fusions

5’-TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG(TTAGGG)n-3’
3’-AAGCCCAAGCCCAAGCCCAAGCCC

TIN2

TRF1

TRF2/Rap1

MRN

ATR

ATM

No chromosome fusions
CtIP is required for A-NHEJ mediated repair of telomeres lacking Tpp1-Pot1a
Naturally shortened telomeres are repaired independent of the C-NHEJ pathway

G4 mTerc−/− 53BP1−/− mice
G4 mTerc−/− 53BP1+/− mice

<table>
<thead>
<tr>
<th></th>
<th>BM</th>
<th>MEFs</th>
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<tbody>
<tr>
<td>G1 BP1+/−</td>
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<td>0.0</td>
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<tr>
<td>G1-G2 BP1+/−</td>
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<td>G3-G4 BP1+/−</td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>G3-G4 BP1−/−</td>
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<tbody>
<tr>
<td>G2 BP1+/+</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>G2 BP1−/−</td>
<td>10.0 ± 1.0</td>
</tr>
<tr>
<td>G4 BP1+/+</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>G4 BP1−/−</td>
<td>5.0 ± 0.5</td>
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Distinct repair pathways depending on how telomeres are uncapped.
Distinct repair pathways depending on how telomeres are uncapped

5'-TTAGGGTTAGGGTTAGGGTTAGGGTTAGGG(TTAGGG)n-3'
3'-AAGCCCAAGCCCAAGCCCAAGCCC

TIN2

TRF1
TRF2/Rap1

MRN
ATM
ATR

C-NHEJ
A-NHEJ

fusions
fusions

natural telomere attrition

CtIP
Acknowledgements

**Pot1a**
Ling Wu
Asha Multani

**Pot1b**
Hua He
Eric Yang

**Rap1**
Rekha Rai
Ming Lei, U of Michigan

**A-NHEJ**
Rekha Rai
Hong Zheng
Philip Carpenter, UT Medical School

**Funding**
NIA
NCI
Susan Komen
Kadoorie Charitable Trust