Caenorhabditis elegans POT-1 and POT-2 Repress Telomere Maintenance Pathways

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ABSTRACT
Telomeres are composed of simple tandem DNA repeats that protect the ends of linear chromosomes from replicative erosion or inappropriate DNA damage response mechanisms. The mammalian Protection Of Telomeres (POT1) protein interacts with single-stranded telomeric DNA and can exert positive and negative effects on telomere length. Of four distinct POT1 homologs in the roundworm Caenorhabditis elegans, deficiency for POT-1 or POT-2 resulted in progressive telomere elongation that occurred because both proteins negatively regulate telomerase. We created a POT-1::mCherry fusion protein that forms discrete foci at C. elegans telomeres, independent of POT-2, allowing for live analysis of telomere dynamics. Transgenic pot-1::mCherry repressed telomerase in pot-1 mutants. Animals deficient for pot-1, but not pot-2, displayed mildly enhanced telomere erosion rates in the absence of the telomerase reverse transcriptase, trt-1. However, trt-1; pot-1 double mutants exhibited delayed senescence in comparison to trt-1 animals, and senescence was further delayed in trt-1; pot-2; pot-1 triple mutants, some of which survived robustly in the absence of telomerase. Our results indicate that POT-1 and POT-2 play independent roles in suppressing a telomerase-independent telomere maintenance pathway but may function together to repress telomerase.

Human somatic cells have finite replicative lifespans and can enter an irreversible cell-cycle arrest, termed senescence, in response to various stresses. Senescence can occur due to progressive shortening of telomeres, which cannot be completely replicated by canonical DNA polymerases (Harley et al. 1990). Telomeres are composed of simple TTAGGG repeats in vertebrates and related sequences in other organisms, such as TTAGGC repeats in Caenorhabditis elegans. To combat telomere erosion, cells can express the enzyme telomerase, which adds de novo telomere repeats to chromosome ends via reverse transcription from an RNA template (Greider and Blackburn 1989). Telomerase is expressed at high levels in germ cells and can be expressed in human somatic cells, but its expression is transient or absent altogether in more differentiated cell types (Kim et al. 1994; Sharma et al. 1995).

The shelterin complex, composed of six mammalian telomere-binding proteins TRF1, TRF2, TIN2, POT1, RAP1, and TPP1, and its associated proteins protect telomeres from nucleases and DNA damage repair mechanisms that can lead to exacerbated telomere shortening or cellular senescence (Diotti and Loayza 2011). Shelterin components maintain telomere homeostasis by positively and negatively regulating telomere length. The double-stranded telomeric DNA-binding proteins TRF1 and TRF2 have been implicated as negative regulators of telomere length, where removal of TRF1 from telomeres or overexpression of TRF2 yielded telomere elongation or erosion, respectively (Smogorzewska et al. 2000; van Steensen and de Lange 1997). TIN2 and TPP1 proteins bridge the interaction between these double-stranded telomere-binding proteins and the single-stranded telomere-binding protein, POT1, and are also considered negative regulators of telomere length as their depletion results in progressive telomere elongation (Kim et al. 1999; Ye and de Lange 2004; Ye et al. 2004).

Human Protection Of Telomeres 1 interacts with single-stranded telomeric DNA via two oligonucleotide/oligosaccharide (OB) folds and is primarily considered a negative regulator of telomere length.
(Kendellen et al. 2009; Veldman et al. 2004; Ye et al. 2004). However, in numerous studies researchers have revealed roles for POT1 in both telomere elongation and telomere protection. POT1 overexpression (Armbuster et al. 2004; Liu et al. 2004) and mutant or splice-variant POT1 expression (Armbuster et al. 2004; Colgin et al. 2003; Kendellen et al. 2009; Liu et al. 2004; Loayza and De Lange 2003) can elicit telomere elongation. In addition, POT1 can inhibit telomere repeat synthesis in the presence of its binding partner TPP1 but promotes telomerase processivity in vitro in its absence (Kellche et al. 2005; Wang et al. 2007).

Both mouse Pot1 homologs promote chromosome end protection, as G-strand overhangs lengthen in Pot1b−/− cells, and end-to-end chromosome fusions occur as a result of telomere deprotection in both Pot1a−/− and Pot1b−/− cells (He et al. 2006, 2009; Hockemeyer et al. 2006, 2008; Wu et al. 2006). However, disparate cellular and telomere phenotypes have been reported. For example, fibroblasts derived from Pot1a−/− mice senesced prematurely in one study (Wu et al. 2006) but not in another (Hockemeyer et al. 2006). In addition, Pot1b−/− cells did not prematurely senesce in one study (Hockemeyer et al. 2006), but mouse embryonic fibroblasts overexpressing an OB-fold Pot1b mutant exhibited early-onset senescence in another study (He et al. 2006). Moreover, telomeres from Pot1b−/− cells have been shown to either shorten or stay the same (He et al. 2009; Hockemeyer et al. 2006, 2008), whereas Pot1a−/− cells exhibited telomere elongation (Wu et al. 2006).

The C. elegans genome is predicted to encode four proteins with OB folds homologous to mammalian POT1, including a single protein with an OB1 fold, POT-1, and three proteins with OB2 folds, POT-2, POT-3, and MRT-1 (Figure 1A) (Meier et al. 2009; Raices et al. 2008). Previous work has illustrated that POT-1, also known as CeOB2, and POT-2, also known as CeOB1, can interact with single-stranded telomeric DNA in vitro (Raices et al. 2008). In addition, this study reported elongated telomeres for both pot-1(tm1620) and pot-2 (tm1400) mutant strains, although pot-1(tm1620) telomeres were distinctive and appeared similar to those of human cells that maintain telomeres by a telomerase-independent telomere replication pathway termed alternative lengthening of telomeres (ALT).

We previously demonstrated that one of four POT1 homologs, MRT-1, is necessary for telomerase-mediated telomere repeat addition in vivo (Meier et al. 2009). Here we investigate additional roles for these C. elegans proteins that contain POT1 OB folds by studying telomere dynamics in pot-1 and pot-2 mutants. We illustrate that both POT-1 and POT-2 are negative regulators of telomerase, indicating a similar and previously unknown role for these proteins in C. elegans telomere biology. We develop a pot-1::mCherry transgene that localizes to telomeres and represses telomerase in vivo. Additionally, we demonstrate a unique role for POT-1 in telomere protection and that POT-1 and POT-2 function non-redundantly to repress a telomerase-independent telomere maintenance pathway.

**MATERIALS AND METHODS**

**Strains**

Unless noted otherwise, all strains were cultured at 20°C on nematode growth medium plates seeded with Escherichia coli OP50. Strains used include Bristol N2 ancestral, CB61 dpy-5(e61) I, YA1059 trit-1(ok410) I, CB402 unc-55(e922) I, CB193 unc-29(e193) I, YA1197 ypln2 (Pdaz-1::pot-1::*mCherry::tbb-2utr), YA1198 ypln3 (Pdaz-1::pot-1::*mCherry::tbb-2utr), YA1024 pot-2(tm1400) II, CB187 rol-6(e187) II, dpy-17 (e164) III, YA1022 pot-1(tm1620) III, unc-32(e189) III, and YA1026 pot-3(ok1530) III.

The pot-1 mutation was outcrossed vs. an outcrossed stock of dpy-17 unc-32, pot-2 and pot-1::*mCherry lines were outcrossed vs. outcrossed stocks of unc-52 or rol-6, respectively. Freshly isolated homozygous F2 lines were established for analysis.

To create pot-1; pot-2 double mutants, a pot-1; unc-52 double mutant and a pot-2; dpy-17 unc-32 triple mutant were first created; phenotypically wild-type F2 progeny of unc-52 / pot-2; pot-1 / dpy-17, unc-32 F1 heterozygotes were selected; and the strains that segregated only phenotypically wild-type F3 progeny were retained for analysis.

To create the tri-1; pot-1 and tri-2; pot-2 double mutants, dpy-5; unc-55; pot-1, dpy-3; unc-55; pot-2, tri-1, dpy-17 unc-32, and tri-1; unc-52 mutants were generated. Phenotypically wild-type F2 progeny of dpy-5; unc-55 / tri-1; tri-1 / dpy-17 unc-32 or dpy-5; unc-55 / tri-1; pot-2 / unc-52 F1 heterozygotes were selected, and strains that segregated only phenotypically wild-type F3 progeny were retained for analysis.

To create the tri-1; pot-2; pot-1 triple mutants, tri-1; pot-2; dpy-17 unc-32 and tri-2; unc-52; pot-1 triple mutants were first created, phenotypically wild-type F2 progeny were selected from tri-1; pot-2 / unc-52; pot-1 / dpy-17 unc-32 heterozygotes, and the strains that segregated only phenotypically wild-type F3 progeny were retained for analysis.

To place the ypln2 pot-1::*mCherry transgene into a pot-1 mutant background, ypln2; dpy-17 unc-32 hermaphrodites were crossed to rol-6 / +; pot-1 / dpy-17 unc-32 males, and phenotypically wild-type F2 progeny were singed from rol-6 / ypln2; pot-1 / dpy-17 unc-32 F1, and F2 that segregated only phenotypically wild-type F3 progeny were retained for analysis. ypln2 pot-1::*mCherry was placed into the pot-2 mutant background analogously, where ypln2 pot-1::*mCherry unc-52 hermaphrodites were crossed to rol-6 / +; pot-2 / unc-52 males and phenotypically wildtype F2 progeny were selected.

To create a pot-1::*mCherry strain that expressed GFP::Histone H2B from the transgene insertion ruls32, N2 males were crossed to hermaphrodites of the strain TH32 unc-119 ed3; dds16[bgl-1::GFP + unc-119(+)] ruls32[unc-119(+)] pie-1::GFP::H2B (Desai et al. 2003), and the resultant F1 males were crossed with pot-1::*mCherry hermaphrodites. F1 cross-progeny were singed and allowed to self-fertilize, F2 progeny were singed from animals heterozygous for both ruls32[unc-119(+)] pie-1::GFP::H2B and pot-1::*mCherry, and F3 progeny homozygous for both transgenes were selected.

**Terminal restriction fragment length analysis**

C. elegans genomic DNA was isolated using Gentra Puregene reagents (QIAGEN), digested with HindIII enzyme (NEB), and separated on a 0.6% agarose gel at 1.5 V/cm. Southern blotting was performed using the DIG Wash and Block Buffer Set (Roche) following the manufacturer’s instructions. A telomere probe, corresponding to the C. elegans telomeric repeat TTAGGG, was synthesized and labeled with digoxigenin (DIG)-dUTPs using the PCR DIG Probe Synthesis Kit (Roche) following the manufacturer’s instructions.

**Telomere erosion rate calculation**

The sizes of individual telomere bands, between 2 and 6 kb, that could be clearly followed for consecutive generations (and were distinct from bands corresponding to neighboring telomeres or to interstitial telomeric tracts) were calculated using semi-log graphs of molecular marker size and distance traveled from the well (Ahmed and Hodgkin 2000; Boerckel et al. 2007; Lowden et al. 2008; Meier et al. 2006, 2009). Data are presented as the mean ± SD.

**Transgene construction**

All transgene constructs were made using the Mos1-mediated single-copy insertion system that allows for the incorporation of a single
copy of a transgene into one specific locus in the C. elegans genome (Frokjaer-Jensen et al. 2008). The \textit{pot-1::mCherry} transgene was constructed using the Invitrogen Gateway Cloning kit using the positive selection marker \textit{Cb-unc-119(+)}, a germline-specific promoter daz-1, full-length genomic \textit{pot-1} sequence lacking a stop codon, \textit{mCherry} sequence, and the \textit{tbb-2} 3' UTR. An extrachromosomal array consisting of this construct, \textit{Pglh-2::Mos1 transposase}, and three fluorescent \textit{mCherry} negative selection markers was introduced into \textit{Mos-1(ttTi5605); unc-119} worms via microinjection into the germline of young adults. Progeny of injected animals were screened for loss of the Unc phenotype and for the presence of \textit{mCherry} fluorescence, suggesting successful transformation of the injected extrachromosomal array. Lines with successful transformants were further propagated and progeny were screened for loss of co-injection \textit{mCherry} fluorescence markers but continued rescue of the Unc phenotype, indicating successful integration of the construct. Genomic DNA prepared from these lines was tested by PCR and DNA sequencing to confirm the presence of a single-copy insertion in the \textit{Mos-1} insertion site on chromosome II. The \textit{unc-119} mutation was removed from transgenic strains prior to analysis by crossing with \textit{rol-6/+} males, singling non-Rol, non-Unc F2 from F1 with Rol F2, choosing F2 that lacked \textit{unc-119} homozygotes, and selecting against \textit{rol-6}. Single-copy transgene insertions were designated \textit{ypIn2 (Pdaz-1::pot-1::mCherry::tbb-2utr)} and \textit{ypIn3 (Pdaz-1::pot-1::mCherry::tbb-2utr)}.
which we refer to below as pot-1::mCherry.B and pot-1::mCherry.C, respectively.

**DAPI staining**

One-day-old adult worms were soaked in 150 μL of a 400 ng/mL DAPI in ethanol solution for 30 min or until evaporated, rehydrated in 2 mL of M9 solution overnight at 4°C, and mounted in 5 μL of fresh NPG/glycerol medium. Chromosome counts were performed under ×100 magnification and a 359 excitation wavelength using a Nikon Eclipse E800 microscope.

**POT-1::mCherry foci quantification**

Live 1-day-old adult worms were mounted onto 2% agarose pads in 5 μL of tetramisole and Z stacks were taken within 2 hr of mounting under ×100 magnification and a 595-nm excitation wavelength using a Nikon Eclipse E800 microscope. Foci from individual nuclei were quantified by manually scanning through compiled Z stacks.

**C-circle quantification**

The C-circle amplification assay was performed as previously described (Henson et al. 2009) with the following modifications: (1) the 96-nucleotide oligomer control was generated with a C. elegans telomeric sequence (5′ CCCATATCCTAA(GCCTAA)9 CCTCAATTC CC 3′); (2) the DNA was resolved on an agarose gel and normalized by ethidium bromide staining, and amplified DNA was dot blotted onto a neutral nylon membrane (GE Healthcare Life Sciences) and probed with a telomeric G strand (TTAGGG)3 oligo conjugated to DIG at 37°C; and (3) the membrane was washed as described for the DIG Wash and Block Buffer Set (Roche) at 37°C and developed with ECF reagent (GE Life Sciences). Fluorescence signals were collected with a Typhoon Trio scanner (GE Life Sciences) and quantified with ImageQuant TL software (GE Life Sciences) using edge subtraction.

**RESULTS**

**POT-1 and POT-2 are negative regulators of telomere extension in vivo**

We obtained strains harboring the deletions pot-1(tm1620) or pot-2(tm1400) from Shohei Mitani and verified the presence of homozygous deletions in these strains using the polymerase chain reaction. Southern blotting revealed long telomeres for genomic DNA isolated from pot-1 or pot-2 mutant strains that were propagated for varying numbers of generations (Figure 1B). In contrast, the pot-3(ok1530) deletion did not have an overt effect on telomere length (Figure 1B). Outcrossing of pot-1 or pot-2 mutations for 15 generations as heterozygotes, followed by isolation of homozygous mutant pot-1 or pot-2 strains, revealed normal telomere lengths in early generations followed by progressive telomere elongation (Figure 1C). Therefore, the telomere elongation phenotypes caused by pot-1 and pot-2 mutations are recessive and can be eliminated if the mutations are maintained as heterozygotes.

Telomeres from pot-1 and pot-2 mutant strains had qualitatively similar dynamics, suggesting that POT-1 and POT-2 may perform similar functions at telomeres. The rapid appearance of smeary, long telomeres, assessed from numerous, outcrossed lines of pot-1 and pot-2 single mutants or pot-1 or pot-2 mutants, precluded measurement of telomere elongation rates with errors of <100 bp/generation, although telomere elongation was qualitatively similar among the three genotypes (Figure 1, C and D; supporting information, Figure S1).

To confirm that the pot-1(tm1620) mutation was responsible for the telomere elongation phenotype of outcrossed pot-1 strains, single-copy transgenes designed to express wild-type POT-1 fused to a fluorescent mCherry protein at its C terminus were created, outcrossed nine times, and crossed into a pot-1(tm1620) background that had been outcrossed 30 times. In contrast to pot-1(tm1620) mutants (Figure 1C), telomere lengths in independent pot-1::mCherry, pot-1 (tm1620) strains remained constant over many generations (Figure 1E), indicating that the progressive telomere elongation phenotype of pot-1(tm1620) mutants is caused by the pot-1 deletion rather than a tightly linked mutation. Moreover, telomeres did not progressively lengthen or shorten for independently outcrossed pot-1::mCherry strains in a wildtype pot-1 background, indicating that the POT-1::mCherry fusion protein does not perturb the ability of endogenous POT-1 to regulate telomere length (Figure 1F). However, bulk telomere length was slightly longer than wildtype for strains containing a pot-1::mCherry transgene (Figure 1, D and F).

**POT-1 foci at C. elegans telomeres in vivo**

Live imaging of animals possessing pot-1::mCherry transgenes revealed strong punctate POT-1::mCherry foci within the nuclei of sperm, some oocytes, and at the nuclear periphery throughout the rest of the germline (Figure 2A–C). POT-1::mCherry foci could be robustly quantified in meiotic pachytene nuclei near the bend of germline arms, where the six homologous chromosomes of C. elegans are synapsed and in late stages of meiotic recombination (Dernburg et al. 1998). Analysis of independent pot-1::mCherry transgene insertions, pot-1::mCherry.B and pot-1::mCherry.C, revealed approximately 12 foci per pachytene nucleus (11.8 ± 0.1; 11.9 ± 0.1; Figure 2G), which could plausibly correspond to chromosome termini of the six paired homologous chromosomes (Figure 2, D and F). Slightly fewer than the expected mean number of telomeric foci were observed, likely due to telomeres that were occasionally near one another within a nucleus, precluding them from being distinguished as distinct foci.

Immunoprecipitation experiments have previously shown that a POT-1::HA fusion protein can interact with telomeric DNA in C. elegans (Raices et al. 2008). To confirm that POT-1::mCherry foci occurred at telomeres, we used the well-characterized end-to-end chromosome fusions ypT24 and ypT28, which were isolated from C. elegans strains that were deficient for telomerase and then crossed onto telomerase-positive genetic backgrounds. These chromosome fusions were created from two chromosome ends that had lost all (TTAGGG)n telomeric repeat sequences as well as several thousand base pairs of subtelomeric DNA prior to being joined together (Lowden et al. 2008, 2011). Strains homozygous for ypT24 and ypT28 X-autosome chromosome fusions harbor five homologous chromosomes and 10 chromosome termini (Figure 2F), and these chromosome fusions can be stably maintained in C. elegans due to the presence of holocentric chromosomes. ypT24 and ypT28 chromosome fusions were crossed with pot-1::mCherry to create pot-1:: mCherry, ypT24 and pot-1::mCherry, ypT28 strains, and quantification of POT-1 fluorescent foci in these strains revealed approximately 10 meiotic foci per nucleus (10 ± 0.1; 9.9 ± 0.1; Figure 2G), indicating that POT-1::mCherry foci correspond to discrete chromosome termini that are not clustered in meiotic pachytene nuclei. In addition, POT-1::mCherry foci were observed at termini of some condensed chromosomes in diakinesis-stage oocyte nuclei, where chromosomes were marked by histone H2B::GFP expression (Figure 2H). However, oocyte nuclei displayed reduced numbers of POT-1::mCherry foci in comparison with pachytene nuclei, and high-resolution images of POT-1::mCherry foci at both ends of a bivalent were not observed.
in oocytes. POT-1::mCherry expression became attenuated and more
diffuse in the oocyte closest to the spermatheca.

Our results demonstrate that POT-1 localizes to telomeres as
small, quantifiable, nuclear domains and is unlikely to form foci at
any other segment of the *C. elegans* genome. To our knowledge, this
is the first demonstration that stable genome rearrangements can be
employed to show that a telomere binding protein specifically
interacts with telomeres in *vivo*. Telomere clustering has been reported as
chromosomes pair during meiosis (Bass et al. 1997; Cooper et al. 1998;
Schertman et al. 1996; Yamamoto and Hiraoka 2001), a process that
occurs in transition zone nuclei of the *C. elegans* germline. POT-1::
mCherry foci became diffuse and were rarely discrete at this stage of
germ cell development (Figure S2), possibly due to rapid chromosome
movements as chromosomes pair.

In contrast to previous results suggesting different functions for
POT-1 and POT-2 (Raices et al. 2008), the lack of a qualitative additive
telomere elongation phenotype for pot-1; pot-2 double mutants suggested a common function for their gene products (Figure 1D;
Figure S1). Therefore, we assessed whether the telomeric localization of
POT-1 was affected by POT-2 by quantifying POT-1::mCherry foci in live pot-1::mCherry, pot-2(tm1400) animals. Approximately 12 foci
per meiotic pachytene nucleus were observed when pot-2 was mutant
(12 ± 0.1), and the POT-1::mCherry localization pattern was qualita-
tively similar throughout the germline in wild-type and pot-2 mutant
backgrounds (Figure 2, E and G). Thus, POT-2 did not have an
obvious effect on the telomeric localization of POT-1.

The distal portion of the *C. elegans* germline is composed of a popu-
lation of proliferating mitotic cells (Cinquini et al. 2010). In contrast
to cells arrested in meiotic pachyten, quantification of POT-1::
mCherry foci in mitotic nuclei revealed an average of 18.9 foci per
nucleus (± 2 SD). We observed a broader range of foci per nucleus in
the mitotic region (16–23, n = 31), in part due to the smaller size and
denser clustering of the nuclei, which precluded the more precise
resolution of POT-1::mCherry foci that was possible in large pachy-
tene nuclei. As some mitotic nuclei displayed less than 24 POT-1::
mCherry spots, weak telomere clustering is likely to occur in mitotic
cells of *C. elegans*. However, the presence of 20–23 spots in some
nuclei suggested that mitotic telomere clustering could either be tran-
sient or could vary with the cell cycle. Our pot-1::mCherry transgene
was only expressed in germ cells, because it was driven by the germ
cell-specific pgl-3 promoter. Future analysis of telomere behavior in
somatic cell types may be an interesting line of investigation.

**POT-1 and POT-2 repress telomerase activity**
**at telomeres**

To ascertain whether the telomere elongation phenotype of pot-1 and
pot-2 mutants is mediated by telomerase, we crossed the *pot-1* and
pot-2 mutations into a telomerase-deficient background by construct-


![](image)

**Figure 2** POT-1::mCherry localizes to telomeres as punctate foci independent of POT-2. Live imaging of pot-1::mCherry strains
revealed germine-specific expression, including meiotic nuclei (A, B; solid brackets), mitotic nuclei (A; dashed brackets), oocytes (B; arrow-
heads), and sperm (C; arrow). (D) Representative image of pot-1::
mCherry. (E) Representative image of pot-1::mCherry; pot-2(tm1400).
(F) A representation of six *C. elegans* chromosomes in wild-type and in
two strains harboring end-to-end chromosomal fusions (ypT24 and
ypT28). Red circles at chromosome termini represent telomeres. (G)
POT-1::mCherry foci were quantified in the meiotic nuclei of two in-
dependent wild-type strains, pot-1::mCherry.B (n = 83) and C (n = 54),
in a pot-2(tm1400) mutant strain (n = 56), and in the strains ypT24 (n = 30) and ypT28 (n = 51). The close paring of both sister chromatids and
homologous chromosomes in *C. elegans* oocytes precludes the resolu-
tion of telomeres from distinct homologous chromosomes and in-
stead reveals 12 or 10 telomeric spots in wildtype or fusion strains,
instead of 24 or 20, respectively. (H) Representative images of live pot-
1::mCherry, GFP::histone animals demonstrate POT-1::mCherry local-
ization at chromosome ends.
each cell division. Previous analysis of strains that are deficient for independent alleles of \textit{trt-1}, for mutations in three additional \textit{C. elegans} genes that are required for telomerase-mediated telomere maintenance, or for double mutants corresponding to \textit{trt-1} and the former genes, has revealed consistent rates of telomere erosion of \textasciitilde120 bp per generation for every genotype (Ahmed and Hodgkin 2000; Boerckel et al. 2007; Lwden et al. 2008; Meier et al. 2006, 2009). We asked whether POT-1 or POT-2 affected the rate of telomere erosion in the absence of telomerase by quantifying telomere shortening for \textit{trt-1}; \textit{pot-1} and \textit{trt-1}; \textit{pot-2} double mutants, for \textit{trt-1}; \textit{pot-2}; \textit{pot-1} triple mutants and for \textit{trt-1} single mutant controls. An enhanced telomere erosion rate was observed for \textit{trt-1}; \textit{pot-1} mutants (159 \textpm 12 bp/generation) in comparison to \textit{trt-1} single mutants (122 \textpm 6 bp/generation; Figure 3D) or \textit{trt-1}; \textit{pot-2} double mutants (123 \textpm 9 bp/generation; Figure 3E). Therefore, POT-1, but not POT-2, protects telomeres from exacerbated erosion in the absence of telomerase. Moreover, \textit{trt-1}; \textit{pot-2}; \textit{pot-1} telomeres shortened at a similar rate (168 \textpm 9 bp/generation) to \textit{trt-1}; \textit{pot-1} telomeres, indicating that POT-2 does not have an obvious telomere protection function in the absence of POT-1 (Figure 3E).

To study the effects of the modestly exacerbated telomere erosion observed in \textit{pot-1} mutant strains that are deficient for telomerase, the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{POT-1 and POT-2 negatively regulate telomerase-mediated telomere repeat addition. DNA collected from consecutive generations of (A) \textit{trt-1}; \textit{pot-1}, (B) \textit{trt-1}; \textit{pot-2}, (C) \textit{trt-1}; \textit{pot-1}; \textit{pot-2}, and (D) \textit{trt-1} mutant strains was submitted to terminal restriction fragment length analysis. (E) To measure shortening rates, telomeres that were 2–6 kb in size and could be accurately scored for changes in length were examined. Error bars represent the SEM, and \textit{P} values were determined by the Student’s \textit{t}-test. (F) Six animals per strain (\textit{n} = 40) of the indicated genotypes were passaged weekly until sterility, where one week indicates two generations of growth. Dashed line to the right of the blots indicates internal telomeric sequences.}
\end{figure}
onset of sterility (senescence) was quantified for \textit{trt-1}; \textit{pot-1} double mutants and \textit{trt-1}; \textit{pot-2}; \textit{pot-1} triple mutants (n = 40 independent lines per strain). Both \textit{trt-1}; \textit{pot-1} double mutants (26.8±1.3 generations) and \textit{trt-1}; \textit{pot-2}; \textit{pot-1} triple mutants (≥44 ± 3.6 genera-
tions) exhibited longer trans-generational lifespans (the number of generations until sterility) in comparison to \textit{trt-1} mutant controls (16.7 ± 0.4 generations; P < 0.001; Student’s t-test), despite their faster rates of telomere erosion (Figure 3F). In addition, \textit{trt-1}; \textit{pot-2}; \textit{pot-1} triple mutants exhibited a significantly longer average trans-generational lifespan than the \textit{trt-1}; \textit{pot-1} double mutants (P < 0.001; Student’s t-test), and some did not senesce (Figure 3F). Thus, although \textit{POT-1} mildly represses telomere shortening in the absence of telomerase, deficiency for \textit{pot-1} or both \textit{pot-1} and \textit{pot-2} failed to enhance the onset of senescence in the absence of telomerase. This increased trans-generational lifespan is consistent with recent reports that either \textit{POT-1} or \textit{POT-2} can suppress the telomerase-independent telomere maintenance mechanism termed ALT (Cheng et al. 2012; Lackner et al. 2012).

It has been previously reported that strains deficient for \textit{pot-1} or \textit{pot-2} with long telomeres display high levels of circular telomeric DNA (Raices et al. 2008). Further, mammalian cells that use the ALT telomere maintenance pathway possess high levels of telomeric C-circles, an established marker of ALT (Henson et al. 2009), and \textit{C. elegans} \textit{trt-1}; \textit{pot-1} ALT strains display increased levels of telomeric C-circles in comparison with wild type (Lackner et al. 2012). We observed wild-type levels of telomeric C-circles in early-generation \textit{pot-2} strains with short telomeres, and large 5- to 7-fold increases in C-circles in late-generation \textit{pot-2} strains with long telomeres (Figure 4). Previously established \textit{trt-1}; \textit{pot-2} ALT strains with telomeres of normal lengths (Cheng et al. 2012) displayed little or no increases in C-circle formation, indicating that the high levels of C-circles in late-generation \textit{pot-2} strains could require the presence of extremely long telomeres and possibly the activity of the telomerase reverse transcriptase. Consistent with the notion that telomerase could contribute to C-circle formation, \textit{trt-1}; \textit{pot-1} ALT strains with either short or very long telomeres displayed modestly elevated levels of C-circles (Figure 4C). Our data suggest that elevated C-circle levels could contribute to ALT, although the greatest levels of C-circles were observed for late-generation \textit{pot-2} that possess long telomeres and were wild type for telomerase.

**DISCUSSION**

\textit{POT-1} is a multifunctional telomere capping protein, and the presence of four \textit{C. elegans} genes with homology to human \textit{POT1} OB folds provides an opportunity to further elucidate the functions of \textit{POT1} in telomere biology. Here we show that \textit{C. elegans} \textit{POT-1} and \textit{POT-2} single-stranded telomere-binding proteins negatively regulate telomerase-mediated telomere repeat addition. In addition, abrogation of telomerase activity in \textit{pot-1} or \textit{pot-2} mutants resulted in progressive telomere erosion. In vitro studies have previously shown that \textit{POT-1} preferentially interacts with single-stranded G-rich telomeric DNA, whereas \textit{POT-2} interacts with single-stranded C-rich telomeric DNA (Raices et al. 2008), suggesting that these proteins may play distinct roles at telomeres. Qualitatively similar telomere elongation dynamics for \textit{pot-1} and \textit{pot-2} mutants, and for \textit{pot-1}; \textit{pot-2} double mutants, suggest that these proteins may function in a similar manner to repress telomerase. Our data do not allow us to distinguish whether \textit{POT-1} and \textit{POT-2} function at distinct steps to repress telomerase or if this occurs via a \textit{POT-1}/\textit{POT-2} heterodimer that possesses both \textit{OB1} and \textit{OB2} folds and could structurally resemble canonical \textit{POT1} proteins (Figure 5A).

We provide evidence for a distinct role for \textit{POT-1} in \textit{C. elegans} telomere biology, as deficiency for \textit{pot-1} but not \textit{pot-2} modestly enhanced the rate of telomere erosion in \textit{trt-1} mutants, suggesting a telomere capping function of \textit{POT-1}. \textit{POT-1} is the sole \textit{C. elegans} protein with an \textit{OB1} fold (Figure 1A) and can interact with non-terminal segments of single-stranded telomeric oligonucleotides in vitro (Raices et al. 2008). Thus, \textit{POT-1} may be well positioned to prevent resection of the 5’ end of the C-rich strand of the telomere. Consistent with our observations, mammalian \textit{POT1} has been shown to protect the 5’ end of the telomeric C-strand, which could be subjected to aberrant processing or resection in the absence of \textit{POT1} (Hockemeyer et al. 2005). In contrast, the \textit{OB2} fold of \textit{POT-2} is predicted to interact with the 3’ end of single-stranded telomeric overhangs (Raices et al. 2008), which could be less relevant to protection or processing of telomeres in the absence of telomerase.

Deficiency for \textit{pot-1} delayed the senescence phenotype of telomerase mutants, even though a modestly faster rate of telomere erosion occurred when \textit{pot-1} was deficient. Recent independent reports have indicated that deficiency for \textit{pot-1} or \textit{pot-2} can promote the
telomere-independent telomere maintenance pathway ALT in 

trt-1

mutants (Cheng et al. 2012; Lackner et al. 2012). POT-1 has been previously shown to repress C-circle formation (Raices et al. 2008), a bona fide marker of ALT, and here we show the same function for POT-2 (Figure 4), suggesting that DNA replication intermediates relevant to ALT may occur in animals lacking either of these proteins. We previously observed an ALT phenotype that allows telomerase mutants to escape senescence indefinitely, but only when hundreds of animals were transferred weekly (Cheng et al. 2012). In the present study, we transferred only 6 animals once a week, which we expected might preclude the onset of a full-blown ALT phenotype. We observed temporary extension of trans-generational lifespan for trt-1 strains deficient for pot-1. However, a subset of trt-1; pot-2; pot-1 triple mutants strains survived indefinitely when 6 larvae were transferred (Figure 3F). This indefinite survival phenotype, in conjunction with a longer trans-generational lifespan of trt-1; pot-2; pot-1 triple mutants in comparison to trt-1; pot-1 double mutants (Figure 3F), is consistent with our previous observation that POT-1 and POT-2 have independent roles in repressing a telomerase-independent telomere replication pathway (Figure 5B; Cheng et al. 2012), which may become fully engaged to robustly drive ALT in early generations (Figure 5B; Cheng et al. 2012), which may become fully engaged to robustly drive ALT in early generations (Figure 5B; Cheng et al. 2012). POT-1 has been previously shown to repress C-circle formation (Raices et al. 2008), a bona fide marker of ALT, and here we show the same function for POT-2 (Figure 4), suggesting that DNA replication intermediates relevant to ALT may occur in animals lacking either of these proteins. We previously observed an ALT phenotype that allows telomerase mutants to escape senescence indefinitely, but only when hundreds of animals were transferred weekly (Cheng et al. 2012). In the present study, we transferred only 6 animals once a week, which we expected might preclude the onset of a full-blown ALT phenotype. We observed temporary extension of trans-generational lifespan for trt-1 strains deficient for pot-1. However, a subset of trt-1; pot-2; pot-1 triple mutants strains survived indefinitely when 6 larvae were transferred (Figure 3F). This indefinite survival phenotype, in conjunction with a longer trans-generational lifespan of trt-1; pot-2; pot-1 triple mutants in comparison to trt-1; pot-1 double mutants (Figure 3F), is consistent with our previous observation that POT-1 and POT-2 have independent roles in repressing a telomerase-independent telomere replication pathway (Figure 5B; Cheng et al. 2012), which may become fully engaged to robustly drive ALT in small populations of animals when both pot-1 and pot-2 are deficient. We observed that early generation trt-1; pot-1 or trt-1; pot-2; pot-1 mutants had longer initial telomere lengths than trt-1 single mutants (Figure 3, A, C, and D), so initial telomere length may be largely responsible for the extended trans-generational lifespans of the double or triple mutants. Given that well-outcrossed pot-1 or pot-2 mutations with short telomere lengths were employed to establish these strains, we speculate that creation of trt-1 strains that are deficient for pot-1 or for both pot-1 and pot-2 promotes a telomerase-independent ALT-like pathway that rapidly extends telomeres in early generations (Figure 5C), but then dissipates allowing late-onset senescence to occur in most strains (Figure 3F).

The modest and progressive effects of deficiency for pot-1 or pot-2 are at odds with rapid and severe telomere phenotypes that occur in the presence of telomerase for S. pombe pot1 mutants (Baumann and Cech 2001), for P. patens pot1 mutants (Shakirov et al. 2010), and for expression of C-terminally truncated Pot2 in Arabidopsis (Shakirov et al. 2005, 2007). More modest effects have been observed when Pot1 was abrogated in mammalian cells (Hockemeyer et al. 2006, 2004). The four C. elegans POT1 homologs may each possess one or more functions of ancestral POT1, allowing their roles in telomere biology to be studied in detail. Finally, our pot-1:mCherry transgene allows for chromosome termini to be observed in living worms and may provide a useful tool for future studies of telomere and chromosome biology in C. elegans.

ACKNOWLEDGMENTS

We thank A. Desai for TH32, the National Bioresource Project for the Experimental Animal C. elegans (Shohei Mitani) for pot-1(tm1620) and pot-2(tm1400), and members of the Ahmed laboratory for discussion and critical reading of the manuscript. Some strains were provided by the Caenorhabditis Genetics Center, which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). This research was supported by NIH grant GM066228 (to S.A.).

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Figure 5 Model for interactions of POT-1 and POT-2 with telomerase and ALT. (A) POT-1 and POT-2 may repress telomerase-mediated telomere length maintenance via independent functions in the same process (left) or as a heterodimer (right). (B) In the absence of telomerase, POT-1 and POT-2 independently repress ALT-mediated telomere maintenance. (C) Early-generation telomerase mutants typically possess telomeres of normal lengths (top right), but deficiency for trt-1 and pot-1 (middle right) or for both pot-1 and pot-2 (bottom right) may initiate a rapid yet transient telomerase-independent telomere maintenance process that extends telomeres (length of double-stranded telomeric duplex is not drawn to scale), thereby allowing for an extended number of generations prior to senescence. A subset of trt-1; pot-2; pot-1 triple mutants (bottom right) may activate an ALT-mediated telomere maintenance pathway, thereby allowing for immortalization in later generations.

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