Caenorhabditis elegans POT-2 telomere protein represses a mode of alternative lengthening of telomeres with normal telomere lengths

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Canonical telomere repeats at chromosome termini can be maintained by a telomerase-independent pathway termed alternative lengthening of telomeres (ALT). Human cancers that survive via ALT can exhibit long and heterogeneous telomeres, although many telomerase-negative tumors possess telomeres of normal length. Here, we report that *Caenorhabditis elegans* telomerase mutants that survived via ALT possessed either long or normal telomere lengths. Most ALT strains displayed end-to-end chromosome fusions, suggesting that critical telomere shortening occurred before or concomitant with ALT. ALT required the 9-1-1 DNA damage response complex and its clamp loader, HPR-17. Deficiency for the POT-2 telomere binding protein promoted ALT in telomerase mutants, overcame the requirement for the 9-1-1 complex in ALT, and promoted ALT with normal telomere lengths. We propose that telomerase-deficient human tumors with normal telomere lengths could represent a mode of ALT that is facilitated by telomere capping protein dysfunction.

PROTECTION OF TELOMERES-2 | telomere maintenance

Telomeres are tandem repeat tracts that cap the ends of linear chromosomes and erode with each cell cycle because of the inability of canonical DNA polymerases to completely replicate chromosome termini (1). However, the ribonucleoprotein telomerase combats this erosion by adding de novo telomeric repeats via reverse transcription (2). Progressive telomere shortening during proliferation of human somatic cells can trigger senescence, which serves as a major tumor suppression mechanism (3). Bypass of senescence and further cell proliferation leads to critical telomere shortening and crisis, resulting in high levels of cell death due to chromosome instability (4). Many tumors overcome this proliferation barrier by activating expression of the telomerase reverse transcriptase. The remaining 10–15% of human tumors do not possess telomerase activity (5, 6).

S. cerevisiae strains deficient for telomerase can survive with long and heterogeneous telomeres composed of amplified telomere repeats (type II survivors) (7, 8). Analogous to type II survivors, long and heterogeneous telomeres occur in almost all in vitro immortalized cell lines and tumor-derived cells lines that are telomerase-negative (9–11). Furthermore, extensive analysis of telomerase activity and telomere length from primary human tumor samples has revealed that certain telomerase-negative tumors possess long and heterogeneous telomeres, defined as alternative lengthening of telomeres (ALT) tumors (9). However, the majority (81/108) of primary tumors lacking telomerase activity have telomeres of normal lengths (9, 12–15). This subset of telomerase-negative tumors may have become neoplastic before exhausting their telomere repeat reserves (9, 16). Alternatively, these tumors could have evolved via the same telomere-driven crisis events that give rise to ALT tumors, followed by activation of a distinct form of ALT that maintains telomeres of apparently normal lengths (9).

Homologous recombination proteins can promote survival in the absence of telomerase. For example, *S. cerevisiae* type II survivors rely on the HR proteins RAD52 and RAD50 for survival (7, 8, 17). In human ALT cells, the MRN homologous recombination complex and the structure-specific endonuclease MUS81, which participates in resolution of recombination intermediates, are required for telomere maintenance and cell survival (18, 19). Recombination may play a role in generating an extrachromosomal telomeric repeat circle, which can be used as a template for rapid telomere elongation via rolling circle replication (20, 21).

To gain a better understanding of ALT, we studied survivors of telomerase deficiency in *C. elegans*. We observed that spontaneous ALT survivors can have long and heterogeneous telomeres or normal telomere lengths, or both. A DNA damage response complex that promotes telomerase activity is required for ALT. Lastly, deficiency for a telomere capping protein promotes a mode of ALT where telomeres are normal in length. These observations suggest that ALT may occur in primary human telomerase-negative tumors that possess telomeres of normal lengths.

Results

Survivors of Telomerase Deficiency in *C. elegans* Can Have Normal Telomere Lengths. To explore the possibility that mutations may promote survival in the absence of telomerase in *C. elegans*, a *trt-1 unc-29* strain that has a deletion in the telomerase reverse transcriptase, *trt-1* (22), and a tightly linked mutation *unc-29* that serves as a visible marker for *trt-1*, was mutagenized with ethylmethanesulfonate (EMS) at generation F11 (23). The *trt-1 unc-29* animals used for this mutagenesis were all derived from progeny of a single freshly outcrossed homozygous *trt-1 unc-29* F2 hermaphrodite whose life began as a zygote with a diploid complement of 24 telomeres, none of which are likely to have been preexisting long telomeres (22). The mutagenized P_0 hermaphrodites, each harboring many EMS-induced mutations per gamete, were singled and allowed to self-fertilize. Each P_0 line produced thousands of starved F2 animals homozygous for some mutations derived from mutagenized P_0 gametes. One hundred and twenty EMS-mutagenized *trt-1 unc-29* P_0 lines and 40 nonmutagenized sibling *trt-1 unc-29* controls were established. Prior experiments with this strain indicated that sterility would occur as a consequence of telomerase deficiency within six generations after mutagenesis, based on an assay where 6 L1 larvae per strain were transferred every two generations (22).

To select for mutations that might promote ALT, agar chunks containing hundreds of mutagenized (m) or control (c) animals per *trt-1 unc-29* strain were transferred monthly for two and a half years, representing ≈40 additional generations. The remaining strains were carefully examined for moderate reproductive capacity, and candidate ALT strains were defined as those where six nonstarved L4 larvae could produce 20–150 progeny. Initially, three strains of 120 initial mutagenized P_0 plates and six strains of 40 nonmutagenized plates met these criteria. After propagation for ≈225 additional generations by

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weekly chunking for >3 yr, only one mutagenized P_0 strain survived, whereas five nonmutagenized control strains persisted, suggesting that ALT may arise spontaneously and that EMS mutagenesis may compromise survival via ALT.

To examine telomere length in the nine initial survivors, Southern blots using a probe for the *C. elegans* telomere repeat sequence $(TTAGGC)_n$ were performed (23). These strains exhibited telomere sizes of <12 kb, typical for wild-type *C. elegans* strains, and strains with long and heterogeneous telomeres of >12 kb, both of which indicate telomerase-independent telomere maintenance. Long and heterogeneous telomeres were accompanied by telomeric DNA in the well, possibly corresponding to complex telomeric structures recalcitrant to entering an agarose matrix (Fig. 1*A*). Continued propagation of several survivor strains with shorter telomeres (<12 kb) resulted in conversion to the long and heterogeneous telomere phenotype in later generations (Fig. 1*B*; strains c3-1s, c1-2s, and c7-4s). Once established, the long and heterogeneous telomere phenotype persisted for many generations, rarely converting to the normal (<12 kb) telomeres lengths (Fig. 1 *A–D*; strains m9-3m and c1-2s). However, telomere lengths of some ALT strains were normal (<12 kb) for many generations and displayed dynamics consistent with periods of continual shortening accompanied by moderate or substantial bursts of telomere lengthening (Fig. 1*C*; strain c8-2s). The c3-1s strain exhibited the most telomere dynamics, switching continuously between normal and long modes over the course of its propagation (Fig. 1 *A–C*).

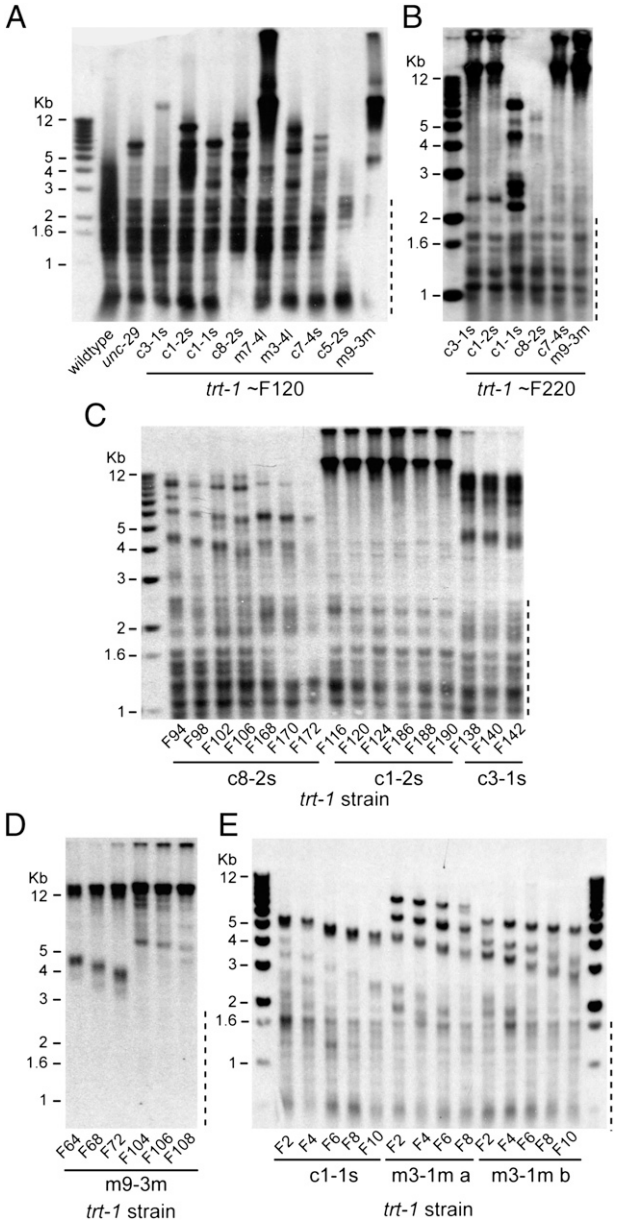

Fig. 1. Telomere dynamics of *trt-1* ALT survivors. Terminal restriction fragment analysis by Southern blotting of DNA from ALT survivors at ≈F120 (*A*) and ≈F220 (*B*). (*C*) Representative ALT strains exhibit normal telomeres (c8-2s and c3-1s) or long and heterogeneous telomeres (c1-2s). (*D*) ALT strain m9-3m exhibits long ALT telomeres and heterogeneous, shortening telomeres. (*E*) ALT survivors exhibit telomere shortening when passaged by transferring 10 L1 larvae every two generations. Dashed lines to the right of each blot correspond to internal telomere sequence tracts.

Although ALT strains could be maintained by transferring >100 animals per generation, these strains became sterile when transferred via population bottlenecks of 6 or 10 animals per generation, typically within 20 generations (Table S1). Southern blotting revealed that small population bottlenecks resulted in progressive telomere shortening, as observed for non-ALT *trt-1 unc-29* telomerase mutants (Fig. 1*E*). Thus, ALT-based telomere maintenance in *C. elegans* requires propagation of >100 animals per generation and may depend on stochastic telomere amplification events that occur in rare animals.

Critical telomere shortening can result in end-to-end chromosome fusion in *C. elegans* telomerase mutants (22, 24). The presence of end-to-end chromosome fusions in ALT strains was assessed by scoring 4',6-diamidino-2-phenylindole (DAPI)-positive spots in oocytes, where wild-type animals have six spots corresponding to the haploid number of six *C. elegans* chromosomes (23). ALT strains commonly exhibited three or four spots, and this number remained stable over many generations (Fig. 2 *A–C* and Table 1). Thus, critical telomere shortening and chromosome end-to-end fusion likely occur before or concomitant with induction of ALT, whereas stable karyotypes are maintained once ALT is established.

To more precisely quantify the ALT-based survival of unmutagenized *trt-1 unc-29* control strains observed above, we repeated the initial experiment with 100 independent lines of *trt-1* under our ALT-favorable conditions where agar chunks containing hundreds of nonmutagenized worms per strain were transferred on a weekly basis (hereafter referred to as "large numbers of worms"). Approximately 20% of the lines survived past 60 generations (=40 wk) (Fig. 3*B*), a growth period that is unattainable for non-ALT *trt-1* strains that were transferred weekly by passaging 6–10 L1 larvae (Fig. 3*A*). These new *trt-1* ALT strains exhibited long and heterogeneous or normal telomere lengths (Fig. 3*C*), analogous to the results observed with the initial ALT strains (Fig. 1*A* and *B*). DAPI staining revealed that all newly derived *trt-1* ALT strains displayed chromosome fusions at levels close to those observed in the original *trt-1 unc-29* ALT strains, which had been passaged for many more generations (Table 2).

9-1-1 DNA Damage Checkpoint Response Complex Is Required for ALT. Components of the 9-1-1 DNA damage checkpoint response complex have been shown to associate with telomeric DNA in human ALT cells (25, 26). The functional significance of the DNA damage response proteins in ALT remains unclear and, in mammals, is difficult to determine because the 9-1-1 complex is essential for cell viability (27). Deficiencies for the *C. elegans* 9-1-1 complex subunits, MRT-2 and HUS-1, and the clamp loader HPR-17, result in viable strains that are defective for telomerase-mediated telomere repeat addition in vivo (23, 28, 29). These mutants can survive for many generations before becoming sterile as a consequence of critical telomere shortening and end-to-end chromosome fusion.

We propagated *mrt-2* and *hpr-17* mutants, including a newly identified allele of *hpr-17*, *yp7* (Fig. S1), in parallel with *trt-1* control strains where large numbers of worms were transferred to assess ALT-mediated survival in the absence of telomerase-mediated telomere repeat addition. Approximately 20% of *trt-1* control strains yielded survivors after 40 generations of growth, whereas all *mrt-2* and *hpr-17* strains became sterile by this time

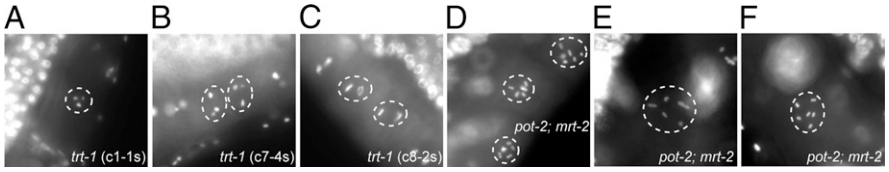

Fig. 2. ALT strains harbor end-to-end chromosome fusions. DAPI photos of representative oocyte nuclei for *trt-1* survivors exhibit three chromosomes (*A* and *B*) or two chromosomes (*C*). *mrt-2; pot-2* survivors exhibit five chromosomes (*D* and *E*) or a wild-type complement of six chromosomes (*D* and *F*). Dashed lines surround chromosomes of individual oocyte nuclei. Wild-type oocytes uniformly display six DAPI-positive spots, comparable to *F* (22, 23, 28).

(Fig. 3*B*; $n = 100$ strains each). Given that the telomeres of *mrt-2, hpr-17,* and *trt-1* single mutants erode at similar rates to those of *trt-1; mrt-2* and *trt-1; hpr-17* double mutants (22, 28), the lack of survivors for *mrt-2* and *hpr-17* suggests that the 9-1-1 complex is required for ALT in *C. elegans*.

POT-2 Represses a Distinct Form of ALT with Telomeres of Normal Lengths. The *C. elegans* genome harbors four genes that encode homologs of mammalian Protection Of Telomeres 1 (POT1), a protein that binds single-stranded telomeric DNA via two oligosaccharide/oligonucleotide (OB) folds, OB1 and OB2 (30–32). *C. elegans* MRT-1, a dual-domain protein comprised of an OB2 fold fused to an interstrand cross-link repair domain, is required for telomerase-mediated telomere repeat addition in vivo (31), whereas worms lacking POT-3, which also harbors an OB2 fold, exhibit wild-type telomeres (32). Two additional POT1 genes, *pot-1* (also known as *CeOB2*) and *pot-2* (also known as *CeOB1*), which encode OB1 and OB2 folds, respectively, have been shown to display elongated telomeres when mutated (24, 32). Moreover, *pot-1* mutants possess heterogeneous telomeres similar to those observed in human ALT cells (32). To test the hypothesis that *pot-1* may promote ALT, we constructed the double mutant strains *trt-1; pot-1*, *trt-1; pot-2*, and *trt-1; pot-3* using well-outcrossed *pot-1*, *pot-2,* or *pot-3* mutant backgrounds that possessed telomeres of wild-type lengths. The double mutant strains were passaged each week by chunking large number of worms ($n = 100$ lines per strain) and by picking population bottlenecks of 10 L1 larvae per strain ($n = \approx 30$ lines per strain). After 70 generations of growth where large numbers of worms were transferred, *trt-1; pot-1* and *trt-1; pot-3* double mutants displayed similar survival frequencies to *trt-1* single mutants, ≈20%, whereas *trt-1; pot-2* strains exhibited a threefold increase in survivorship, ≈65% (Fig. 3*B*). In contrast, population bottlenecks of 10 larvae every two generations yielded very few or no survivors for these strains (Fig. 3*A*). Thus, deficiency for *pot-2* rather than *pot-1* enhances the frequency of ALT where large numbers of worms were transferred. However, *trt-1; pot-1* ALT strains typically displayed five DAPI-positive spots in oocytes, in contrast to four spots in *trt-1* or *trt-1; pot-2* ALT strains (Table 2). We confirmed that the reduced number of DAPI-positive spots in four independent *trt-1; pot-1* ALT strains likely occurred as a consequence of X-autosome telomere-telomere fusions by demonstrating genetic linkage between the left end of the X chromosome and a dominant chromosome nondisjunction phenotype caused by chromosome fusions present in these strains (Fig. S3) (23, 24). Thus, *pot-1* deficiency may modestly reduce the frequency of end-to-end chromosome fusion in ALT strains. This result could indicate a role for wild-type *pot-1* in genesis of chromosome fusions in the absence of telomerase. Alternatively, induction of ALT could be more efficient when it occurs in *trt-1; pot-1* double mutants, such that fewer chromosome fusions occur, even though the frequency of ALT is not increased.

Seventy percent of the *trt-1* and *trt-1; pot-1* survivors exhibited telomeric signals >20 kb, accompanied by signals in the well, whereas the remaining survivors had telomeres of short or normal lengths (Fig. 3 *C* and *D* and Fig. S2). In contrast, 18/18 *trt-1; pot-2* strains possessed telomeres that were predominantly short or normal in length (Fig. 3*D* and Fig. S2). Although longer telomeres were observed in some *trt-1; pot-2* strains, they were shorter than the large bands (>20 kb) observed in *trt-1* and *trt-1; pot-1* strains with long and heterogeneous telomeres (Fig. 3*D*). The increase in the frequency of *trt-1; pot-2* survivors, and their shorter telomere lengths, support a role for *pot-2* deficiency in promoting a mode of ALT that is distinct from that associated with long and heterogeneous telomeres in telomerase-negative human tumors.

Deficiency for *pot-2* Allows for ALT in the Absence of the 9-1-1 Complex. Because the 9-1-1 complex is required for ALT in *C. elegans*, we asked whether it is also required for the predominantly short telomere form of ALT that occurs when *pot-2* is deficient. Although all *pot-2; mrt-2* strains became sterile when propagated in small population bottlenecks (Fig. 3*A*), ≈90% of the lines survived past 60 generations when transferred using large numbers of worms per strain (Fig. 3*B*). Southern blotting revealed a lack of long telomeric signals for *pot-2; mrt-2* ALT strains, confirming that *pot-2* deficiency may promote a specific type of ALT with telomeres of normal lengths (Fig. 3*E*). Normal telomere length was maintained over the course of 20–40 generations for all seven *pot-2; mrt-2* strains examined and for six *trt-1; pot-2* strains A, B, C, D, F, and G, although the *trt-1; pot-2* E strain switched from normal telomere length to long and heterogeneous telomeres (Fig. 3 *F* and *G*).

Because deficiency for the POT-2 telomere binding protein eliminates the requirement of *mrt-2* for survival where large numbers of worms were transferred, the lack of survivors in strains deficient for the 9-1-1 complex or its clamp loader HPR-17 is almost certainly due to the inability of critically shortened telomeres to initiate ALT, as opposed to deficiency for a telomere-independent function of the 9-1-1 complex or HPR-17, such as responding to DNA damage. In addition, the sterility phenotypes of *mrt-2* and *hpr-17* have been addressed in detail and are fully consistent with resulting from telomerase-dependent telomere repeat addition defects (22, 23, 28). Note that although *mrt-2* is required for telomerase-mediated telomere repeat addition in vivo (22), *trt-1* is wild type in *mrt-2* strains, and we cannot exclude the possibility that mutation of *pot-2* allows for infrequent stochastic

Table 1. Reduced chromosome numbers in late-generation ALT survivors

Strain	F40	F50	F80	F220
wildtype	6.0 ± 0.0 (n = 13)	ND	ND	ND
c1-1s	3.9 ± 0.4 (n = 21)	2.8 ± 0.4 (n = 19)	3.0 ± 0.4 (n = 20)	2.8 ± 0.4 (n = 15)
c1-2s	3.8 ± 0.5 (n = 20)	2.9 ± 0.3 (n = 18)	2.9 ± 0.3 (n = 14)	2.9 ± 0.3 (n = 15)
c3-1s	3.8 ± 0.4 (n = 24)	2.8 ± 0.4 (n = 22)	2.9 ± 0.4 (n = 20)	2.9 ± 0.3 (n = 15)
c5-2s	2.9 ± 0.3 (n = 16)	3.1 ± 0.1 (n = 20)	3.0 ± 0.0 (n = 10)	ND
c7-4s	4.2 ± 0.6 (n = 15)	3.0 ± 0.2 (n = 20)	2.9 ± 0.3 (n = 14)	3.1 ± 0.4 (n = 17)
m3-4l	4.8 ± 0.5 (n = 19)	2.7 ± 0.5 (n = 18)	2.9 ± 0.4 (n = 16)	ND
m9-3m	4.8 ± 0.4 (n = 27)	2.9 ± 0.2 (n = 17)	3.0 ± 0.0 (n = 16)	2.8 ± 0.4 (n = 18)

Whole worms were stained with DAPI, and condensed chromosomes were counted in the nuclei of 3–5 oocytes per worm in the indicated number of worms.

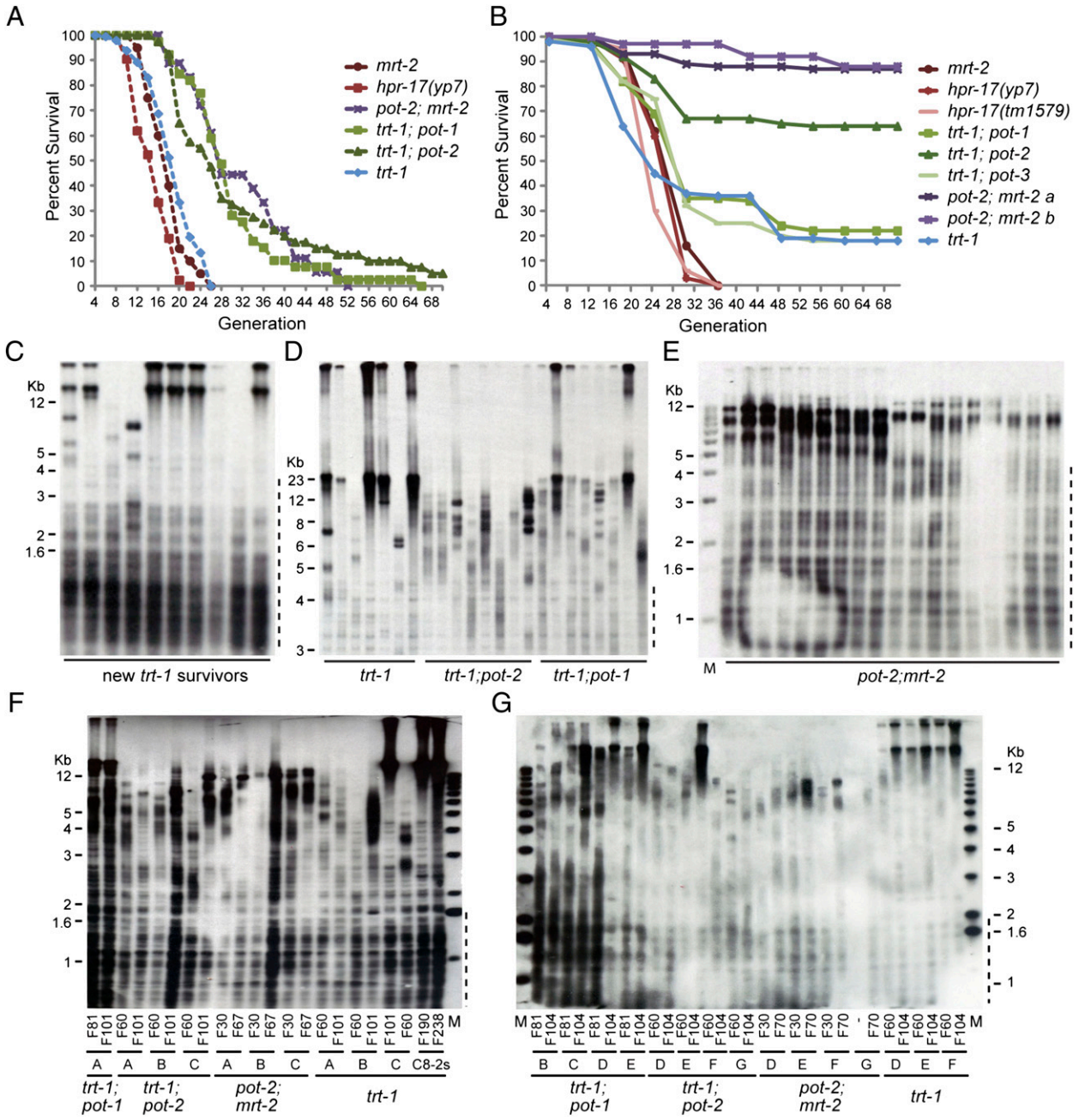

Fig. 3. *pot-2* deficiency promotes a type of ALT with a distinct shorter telomere signature. (*A*) *trt-1* or the 9-1-1 complex single mutants, as well as *pot-1* or *pot-2* double mutants, become sterile if passaged by transferring 10 larvae weekly (n = roughly 30 strains per genotype, two generations per week). (*B*) Single and double mutant strains can survive if chunked weekly (n = 100 plates per genotype, one and a half generations per week). (*C*) New *trt-1 (ok410)* ALT survivors exhibit both long and normal telomeres. (*D*) Generation F60 *trt-1; pot-2* survivors exhibit normal-length telomeres, whereas *trt-1; pot-1* survivors exhibit predominantly long telomeres. (*E*) Generation F30 *pot-2; mrt-2* strains survive exclusively with normal length telomeres. (*F* and *G*) Telomere length maintenance as ALT strains are propagated. Dashed bars to the right of each blot correspond to internal telomere sequence tracts.

access of telomerase to telomeres, thereby promoting survival only where large numbers of worms were transferred. However, because *pot-2; mrt-2* strains fail to survive when subjected to population bottlenecks, and because *trt-1; pot-2* strains display properties in common with *pot-2; mrt-2* strains, such as a high frequency of survival where large numbers of worms were transferred, and maintenance of predominantly normal telomere lengths (Fig. 3), we favor the possibility that late-passage *pot-2; mrt-2* strains survived via ALT when large numbers of worms were transferred.

DAPI staining of *pot-2; mrt-2* survivors revealed that few chromosome fusion events had occurred in these strains (Fig. 2*D*), many of which displayed a wild-type karyotype of six DAPI-positive spots (Table 2 and Fig. 2 *E* and *F*). Thus, when *pot-2* is deficient, MRT-2 may inhibit the efficiency of ALT induction, because chromosome fusions occur in *trt-1; pot-2* ALT strains but rarely in *pot-2; mrt-2* ALT strains.

Discussion

Here, we demonstrate that telomeres of a multicellular organism can be maintained indefinitely in the absence of telomerase. *C. elegans* ALT strains arise spontaneously at frequencies that can be manipulated by several proteins known to function in telomere

Table 2. Telomeric fusions in ALT survivors

Wildtype	Original *trt-1*	New *trt-1*	*trt-1;pot-2*	*trt-1;pot-1*	*pot-2;mrt-2*
6.0 ± 0.0 (*n* = 25)	2.8 ± 0.4 (*n* = 15)	3.8 ± 0.4 (*n* = 13)	3.9 ± 0.3 (*n* = 9)	4.8 ± 0.4 (*n* = 13)	5.8 ± 0.4 (*n* = 11)
	2.9 ± 0.3 (*n* = 15)	3.9 ± 0.3 (*n* = 13)	4.2 ± 0.4 (*n* = 13)	5.2 ± 0.4 (*n* = 13)	5.1 ± 0.3 (*n* = 13)
	2.0 ± 0.3 (*n* = 15)	4.1 ± 0.5 (*n* = 14)	4.4 ± 0.5 (*n* = 13)	4.9 ± 0.3 (*n* = 13)	5.4 ± 0.7 (*n* = 11)
	3.1 ± 0.4 (*n* = 17)	3.3 ± 0.5 (*n* = 14)	4.3 ± 0.5 (*n* = 12)	4.2 ± 0.4 (*n* = 17)	6.0 ± 0.0 (*n* = 11)
	2.8 ± 0.4 (*n* = 18)	3.8 ± 0.4 (*n* = 13)	4.1 ± 0.4 (*n* = 15)	4.9 ± 0.2 (*n* = 17)	5.7 ± 0.5 (*n* = 14)
		4.2 ± 0.4 (*n* = 13)	4.1 ± 0.3 (*n* = 11)	4.9 ± 0.4 (*n* = 14)	5.5 ± 0.5 (*n* = 11)
		3.9 ± 0.4 (*n* = 14)	4.0 ± 0.0 (*n* = 14)	4.9 ± 0.5 (*n* = 15)	5.0 ± 0.4 (*n* = 12)
		3.2 ± 0.4 (*n* = 12)	4.7 ± 0.5 (*n* = 18)	4.9 ± 0.4 (*n* = 14)	5.8 ± 0.4 (*n* = 13)
		3.9 ± 0.4 (*n* = 14)	4.0 ± 0.4 (*n* = 14)	4.2 ± 0.4 (*n* = 17)	5.8 ± 0.4 (*n* = 11)
		3.9 ± 0.3 (*n* = 10)	3.9 ± 0.3 (*n* = 12)	5.0 ± 0.0 (*n* = 12)	6.0 ± 0.0 (*n* = 14)
		4.0 ± 0.0 (*n* = 10)	4.2 ± 0.4 (*n* = 16)	4.9 ± 0.3 (*n* = 10)	6.0 ± 0.0 (*n* = 12)
			4.0 ± 0.0 (*n* = 6)	4.8 ± 0.4 (*n* = 16)	5.7 ± 0.5 (*n* = 10)
			4.1 ± 0.4 (*n* = 15)		6.0 ± 0.0 (*n* = 10)
			4.0 ± 0.0 (*n* = 10)		6.0 ± 0.0 (*n* = 12)
			4.0 ± 0.0 (*n* = 12)		5.9 ± 0.3 (*n* = 13)
					5.8 ± 0.4 (*n* = 12)
					6.0 ± 0.0 (*n* = 12)
					5.8 ± 0.4 (*n* = 13)

Whole worms were stained with DAPI, and condensed chromosomes were counted in the nuclei of 3–5 oocytes in the indicated number of worms. Results for individual strains are presented.

biology. Telomeres of normal lengths were observed in several spontaneous *trt-1* ALT strains. Deficiency for the POT-2 telomere protein in either *trt-1* or *mrt-2* telomerase-defective backgrounds yielded ALT strains with distinctly normal telomere lengths. We propose that telomerase-negative human tumors with telomeres of normal lengths may represent this distinct form of ALT (9), some of which could arise as a consequence of mutations in genes that encode telomere-binding proteins.

Telomerase-independent telomere maintenance that results in telomeres of normal length has been reported for primary and lymphoma cells from mouse telomerase RNA mutants (33), although other studies have revealed long and heterogeneous telomeres in mouse tumors derived in the context of telomerase deficiency (34, 35). One immortalized human cell line, C3-cl6, maintained short and homogeneous telomeres in the absence of telomerase (10). These studies, in addition to many telomerase-negative tumors that have been shown to lack long and heterogeneous telomeres (9, 12–15), support the prospect of an ALT mechanism where telomeres are normal in length rather than long and heterogeneous.

ALT is overrepresented in certain tumors, suggesting that a specific developmental program or cellular niche may promote ALT (16, 36). However, little is known about the genetic basis for ALT in human tumors (37). In yeast, the absence of telomere proteins Rif1 or Rif2, which normally repress telomerase activity, has been shown to promote survival of telomerase-deficient strains with long and heterogeneous telomeres (38). Of two *C. elegans* POT1-related genes that exhibit elongated telomeres when absent, *pot-1* and *pot-2* (24, 32), we show that deficiency for *pot-2* specifically promotes the frequency of survival via ALT, whereas deficiency for *pot-1* results in ALT strains that possess fewer chromosome fusions. These results agree with a previous report suggesting that *pot-1* and *pot-2* have overlapping but distinct biological functions (32). The high frequency of *trt-1; pot-2* survivors is consistent with a role for the POT-2 OB2 fold protein in binding and protecting the 3′ end of a chromosome (32), where ALT could initiate by engaging an illegitimate replication or recombination mechanism (Fig. 4*A*). In addition, ALT strains deficient for *pot-2* had predominantly normal telomere lengths, suggesting that POT-2 could promote bursts of ALT activity that lead to long and heterogeneous telomeres, or that it may function to repress trimming of long telomeres (Fig. 4*A*), which is a natural process that can act on ALT and non-ALT telomeres alike (39).

The 9-1-1 complex and HPR-17 represent a DNA polymerase processivity factor and its clamp loader, which could physically associate with critically shortened telomeres to fulfill their role in telomerase-independent telomere maintenance. The homologous recombination function of these proteins (40) may promote ALT, possibly by facilitating the association of critically shortened telomeres with recombination proteins that have been implicated in ALT in diverse organisms (41). POT-2 may normally repress a recombination or replication intermediate that channels critically shortened telomeres into the ALT telomere maintenance mechanism, thereby promoting ALT in the absence of the 9-1-1 complex (Fig. 4*B*). We conclude that POT-2 functions not only to repress ALT, but also to promote long and heterogeneous telomeres once the ALT pathway is engaged (Fig. 4).

The presence of chromosome fusions in most *C. elegans* survivors suggests that ALT initiation could be an inefficient process, even for *trt-1; pot-2* strains that display a threefold increase in the frequency of ALT. Alternatively, the fusion process itself, or associated levels of DNA damage signaling, may play critical roles in initiation of ALT, which is supported by a model that ALT acts on critically shortened telomeres in *S. cerevisiae* (8). Consistent with this concept, human ALT cells can be derived in vitro only if senescence is bypassed and cells reach crisis, where critically shortened telomeres and chromosome fusions are abundant (9, 16). Because our ALT-favorable conditions required transferring large numbers of animals in all backgrounds, induction and maintenance of ALT may rely on rare

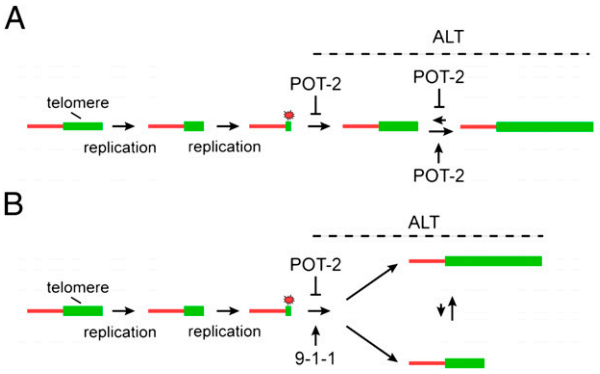

Fig. 4. ALT pathway models. (*A*) POT-2 inhibits induction of ALT and could either repress trimming of long ALT telomeres or promote conversion to long and heterogeneous telomeres. (*B*) The 9-1-1 complex promotes ALT via a mechanism that may be repressed by POT-2.

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stochastic events. The striking lack of chromosome fusions in *pot-2; mrt-2* ALT strains suggests that ALT initiation is more efficient when it occurs in this background, perhaps acting readily on a single initial critically shortened telomere (Fig. 4). However, ALT is not constitutively active in *mrt-2; pot-2* strains, which become sterile because of telomere dysfunction if propagated by transferring small numbers of worms (Fig. 3*A*).

Further analysis of ALT mechanisms may provide deeper insight into the status of telomere maintenance in tumors, which can serve as a prognostic marker for patients (12) and may guide studies relevant to the etiology and treatment of telomerase-negative cancers.

Materials and Methods

Strains. All experiments were conducted at 20 °C under standard culture conditions. The following mutations were used in this study: *dpy-5(e61) I*, *trt-1(ok410) I*, *unc-29(e193) I*, *rol-6(e187) II*, *rol-1(e91) II*, *hpr-17(tm1579) II*, *hpr-17(yp7) II*, *pot-2(tm1400) II*, *unc-52(e444) II*, *pot-1(tm1620) II*, *unc-32(e189) III*, *dpy-18(e364) III*, *mrt-2(e2663) III*, *pot-3(ok1530) III*, *unc-64(e246) III*. *pot-1 (tm1620)* and *pot-2(tm1400)* deletion mutations were outcrossed 15 times versus *unc-32* or *unc-52* marker mutations, which had been crossed three times versus an N2 ancestral male stock. Double mutant strains were then constructed by using *dpy-5 unc-29*, *unc-52*, *unc-32*, *dpy-18*, or *unc-64* as balancers for *trt-1*, *pot-2*, *pot-1*, *mrt-2*, or *pot-3*, respectively. Briefly, *trt-1; unc-52*, *trt-1; unc-32*, *trt-1; unc-64* or *trt-1; dpy-18* strains were crossed with *dpy-5 unc-29; pot-2*, *dpy-5 unc-29; pot-1*, *dpy-5 unc-29; pot-3*, or *dpy-5 unc-29; mrt-2* triple mutant strains, respectively, where the latter pot triple mutant strains were freshly constructed from well-outcrossed *pot*/+ heterozygous males and, therefore, contained wild-type telomere lengths. All genotypes were confirmed by PCR of deletion mutations for *trt-1*, *pot-2*, *pot-1*, or *pot-3*, or scoring radiation hypersensitivity for *mrt-2* (23, 28).

Isolation of ALT Survivors. When telomerase-deficient *C. elegans* mutants are passaged by using the standard Mortal Germline assay developed in our laboratory, which involves passaging six animals to fresh plates weekly, 100% become sterile within 15–30 generations. When *trt-1(ok410)* strains were passaged, weekly or monthly where indicated, by transferring a 0.5 cm × 0.5 cm chunk of agar populated with ≈150–400 animals, ≈20% of the strains survived for hundreds of generations. For initial survivor experiments comparing mutagenized versus nonmutagenized *trt-1* strains, a *trt-1(ok410) unc-29* double mutant strain was used, where the *trt-1* deletion *ok410* is tightly linked to the marker mutation *unc-29*.

Telomere Restriction Fragment Length Analysis. *C. elegans* genomic DNA was digested with HinfI (New England Biolabs), separated on a 0.6% agarose gel at 1.5 V/cm, and transferred onto a neutral nylon membrane (Hybond-N; GE Healthcare Life Sciences). A digoxigenin-dUTP-labeled telomere probe was hybridized and detected as described (23, 28).

DAPI Staining. Adult worms were suspended in an ethanol solution of DAPI (150 μL, 400 ng/mL), and the solution was allowed to evaporate over 30 min. Worms were rehydrated in 2 mL of M9 at 4 °C overnight, mounted in NPG/glycerol medium (23, 28), and scored with a Nikon Eclipse E800 microscope.

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