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C. elegans survivors without telomerase

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In most eukaryotic organisms with a linear genome the telomerase complex is essential for telomere maintenance and, thus, for genomic integrity. Proper telomerase function in stem and germ cell populations counteracts replication-dependent telomere shortening. On the other hand, repression of telomerase expression in most somatic tissues limits the proliferative potential of these cells through the induction of a permanent cell cycle arrest termed senescence upon critical telomere erosion. Thus, senescence, induced by telomere shortening and subsequent DNA damage signaling, is an essential tumor-suppressive mechanism, emphasized by the fact that repression of telomerase is lost in about 90% of cancers, endowing them with unlimited proliferative potential. In 10% of cancers, telomeres are maintained using the recombination-based alternative mechanism of telomere lengthening (ALT). To date, ALT and ALT-like mechanisms have only been described in the context of individual cells such as cancer cells and yeast. Now, several "survivor" strains of the nematode *Caenorhabditis elegans* have been generated that can propagate despite mutations of the telomerase gene. These nematode strains represent the first multi-cellular organism with canonical telomerase that can survive in the absence of a functional telomerase pathway.

Keywords: *C. elegans*, telomere, alternative mechanism of telomere lengthening, telomerase, senescence, *pot-1*, *trt-1*, *pot-2*

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Telomeres are the physical ends of linear chromosomes and are essential for conserving the integrity of the genome. They usually consist of several kilobases of double-stranded G-rich repetitive DNA sequence [$(TTAGGG)_n$ in mammals and $(TTAGGC)_n$ in the nematode *Caenorhabditis elegans*] ending in a shorter single-stranded overhang. Telomeres serve two main functions in the cell: first, they act as protective structures to ensure that the ends of linear chromosomes are not being recognized as points of DNA damage. This protection is achieved in conjunction with specific telomere-binding proteins that bind to the double-stranded or single-stranded portion of the telomere. In mammals, the core telomeric protein complex is called shelterin and is comprised of six members.[1] In *C. elegans*, to date only two telomere-binding proteins have been identified and they both have been shown to bind to the single-stranded overhang portion of the telomere.[2]

Second, telomeres act as a buffer to counteract replication-dependent chromosome shortening. Due to the limitations of DNA polymerases in combination with end processing, telomeres shorten during each replication cycle, since the very 3' end of the template cannot be replicated ("the end-replication problem").[3] Once telomeres become critically short they induce a DNA damage response that can ultimately induce an irreversible cell cycle arrest called senescence. As senescence limits the growth proliferation of human somatic cells, this is considered as an important tumor-suppressive mechanism.[4,5]

In germ and stem cells, the telomerase enzyme counteracts telomere shortening by using its own RNA template and reverse transcriptase to add telomeric repeats to the ends of the chromosomes.[6,7] Thus, telomerase provides these cells with an infinite replicative lifespan. As such, it is not surprising that telomerase, the expression of which is repressed in somatic tissues, is highly upregulated in about 90%

of cancer cells.[8] However, in about 10% of cancers, telomerase remains shut-off and telomeres are maintained using ALT (alternative lengthening of telomeres).[9,10] Whereas ALT was initially referred to as any alternative telomere maintenance mechanism not involving the telomerase enzyme, it is now more commonly used to describe a specific mechanism in mammalian cells. ALT is based on recombination between telomeres,[11] but the exact mechanism is still elusive.[12] Thorough understanding of how ALT works on a molecular level is highly relevant in the context of the potential use of telomerase-inhibitors as anticancer drugs, as these drugs will not be efficient in the 10% of ALT-positive cancers. Furthermore, there is mounting evidence that targeting of telomerase might actually induce ALT in certain cases,[13] emphasizing the need to fully understand both telomere maintenance pathways.

Until now, ALT and ALT-like mechanisms have only been described for cellular systems, such as in the aforementioned tumor cells and yeast. However, recent work from the Ahmed and Karlseder laboratories have now demonstrated that *C. elegans* strains can survive in the absence of a functional telomerase pathway, implicating the involvement of ALT-like mechanisms.[14,15] While there are some differences in the details of these two studies, the emerging overall picture is very similar: mutations in the *C. elegans* telomerase gene (*trt-1*) have no initial impact on the organisms due to a sufficient telomeric sequence buffer. Eventually, progressive telomere erosion over several generations leads to DNA damage signaling and genomic instability, which ultimately results in sterility.[16] However, using differing approaches, the Ahmed and Karlseder labs have now generated nematode strains with mutations in the telomerase gene that have managed to propagate for more than 200 generations and still thrive. These nematode strains represent the first multicellular model for ALT.

In our approach, we exploited the phenotype of a *C. elegans* strain with a mutation in the telomere-binding protein CeOB2/POT-1, since mutation of *pot-1* has been shown to result in increased telomere length heterogeneity reminiscent of human ALT cells.[2] Furthermore, in *pot-1* mutants, we found enriched levels of a species of single-stranded (ss) C-rich telomeric circles,[14] which has been described as a marker of ALT activity in human cells,[17,18] suggesting increased telomeric recombination. When *pot-1/trt-1* double mutant strains were tested for long-term survival by transferring five to six worms every two generations, several survivor lines could be established that have now been propagated for more than 200 generations. All *trt-1* single mutants became sterile during the experiment.

The Ahmed lab initially set out to find suppressor-mutations in *trt-1* mutants that would allow for long-term survival in the absence of a functional telomerase pathway. They initially mutagenized early generation *trt-1* mutants and then tested for long-term survival by transferring chunks of agar containing hundreds of worms over a long period of time. Cheng et al. discovered that after chunking the worms for more than 260 generations, there were more survivor lines in the non-mutagenized *trt-1* negative control (5) than in the mutagenized *trt-1* animals (only one line).

It is important to point out that in both cases the majority of *trt-1*-deficient lines still became sterile after a finite number of generations, most likely due to telomere erosion and genomic instability, and that the emergence of telomerase-negative survivor strains is a rare event. This suggests a mechanism where an adaptation process, likely in the form of additional mutations, takes place that allows the survivor strains to propagate. These mutations might arise through a crisis-like process, which in mammalian cells often precedes the transition of a healthy to a cancerous cell devoid of proper regulation of proliferation. During crisis, cells with critically short telomeres continue to divide, resulting in telomere-driven breakage fusion cycles and ultimately genomic instability. Eventually, cells emerge that have lost checkpoint controls and proliferate rapidly. Accordingly, in our study, we have observed that survivor lines often become almost sterile and then display a sudden recovery over the next generations, pointing toward a selection mechanism where strains emerge that can propagate in the absence of a functional telomerase pathway. The mutation of *pot-1* facilitated this selection process in our hands, since telomeres were already rendered more recombinogenic. In the Ahmed study, the much higher number of nematodes used might have aided the selection of single *trt-1* mutant survivors.

The Ahmed laboratory report a massive increase of telomerase-negative survivors in a strain mutated for the second *C. elegans* telomere-binding protein CeOB1/POT-2, which had initially been suggested to be responsible for regulation of telomerase access to telomeres, since mutations in this gene result in extremely long telomerase-dependent telomeres[2] (data not shown). The results by the Ahmed lab suggest the possibility that mutation of *pot-2* renders telomeres not only longer, but also much more recombinogenic. Indeed, when we tested a *pot-2* mutant strain for C-circles, we found more such circles as compared with *pot-1* mutants, proposing even higher recombination potential, which could facilitate ALT (unpublished data). On the other hand, given the long telomeres in these strains and the fact that Cheng et al. tested the long-term survival of the *pot-2* mutant lines for only 70 generations, it is possible that the strains can propagate longer than the *trt-1* single mutants, simply because of initially longer telomeres, accounting for the large number of survivor strains. This is in line with results from our lab, where *C. elegans* strains that have naturally longer telomeres show a significantly increased long-term survival when *trt-1* is mutated, as compared with *trt-1* mutants with shorter telomeres.[14] Further experiments are needed for a final conclusion in this regard.

Both studies show that telomerase-negative survivors exhibit features also found in human ALT cells, such as telomere length heterogeneity and signs of genomic instability in the form of fused chromosomes. The transcription profiles of survivor strains were surprisingly similar to late generation *trt-1* mutants that eventually became sterile.[14] However, despite chromosome fusions, survivors did not become sterile and managed to propagate, even though the overall fitness of the strains was diminished in comparison

to wild-type animals, emphasized by slower development, less eggs laid and unhatched eggs.

How and why do *C. elegans* strains manage to propagate in the absence of a functional telomerase pathway? There are a few possible explanations why *C. elegans* might be generally less susceptible to the effects of DNA damage and damage signaling. First, apart from germ cells, adult *C. elegans* tissue is post-mitotic and as such cells only need to undergo a finite number of divisions. As a consequence, there is no need for mitotic cell populations that undergo constant proliferation to replenish the organism (such as stem cells). The lower amount of progeny and unhatched eggs in the survivor strains suggest partial germ cell failure, and points to the fact that germ cells could be most susceptible to DNA damage. Second, *C. elegans* has holocentric chromosomes.[19] Consequently, their chromosomes are not dependent on a single centromere for faithful segregation during mitosis and meiosis, and even fused chromosomes can be evenly segregated during cell division,[20] potentially allowing for better selection of mutated, but stable, telomerase-negative strains.

As mentioned, given the rare emergence of survivor strains, it is most likely that additional mutations play an important role. These mutations could be gain-of-function mutations in genes important for DNA recombination events or loss-of-function mutations in important checkpoint genes, and of course a combination of both. It will be exciting to unravel the nature of these mutations, but this is no easy task, given that every mutation would have to be tested for long-term survival in combination with the *trt-1* mutation.

C. elegans survival in the absence of a functional telomerase-pathway points out an interesting evolutionary aspect. In the fruit fly *Drosophila melanogaster*, the telomerase enzyme is absent and telomere maintenance is achieved trough transposition of telomere-specific transposable elements.[21] Both *D. melanogaster* and *C. elegans* belong to the clade of Ecdysozoa and are evolutionary quite distant from mammals and even from some of the more basic common eumetazoan ancestors, such as sea anemones or jellyfish.[22,23] It is tempting to speculate that alternative mechanisms of telomerase maintenance have evolved in this clade independently of mammalian ALT, both as main telomere maintenance mechanism (*D. melanogaster*) and as back-up mechanism in case of telomerase de-activation (*C. elegans*). It will therefore be necessary to describe the telomere maintenance mechanisms for many other yet uncharacterized model systems and potentially point out that various ALT mechanisms are more prevalent than previously thought. Alternatively, one could conclude that *C. elegans* is evolutionarily so far removed from mammals that it might not be a useful model system to study such complex mechanisms as telomere length maintenance. However, despite the evolutionary distance between mammals and nematodes, more work is necessary to identify molecular players that are essential for the ALT-like survival in *C. elegans*, and it will be exciting to see if there is overlap with some of the few identified players of ALT in mammals.

As for ALT in general, it will be exciting to uncover whether ALT is a mechanism that can simply be "switched on" as a back-up when telomerase-mediated telomere maintenance fails, whether ALT could even work in conjunction with telomerase, or if ALT happens serendipitously as a combination of additional mutations in telomerase-negative strains.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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