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Organismal propagation in the absence of a functional telomerase pathway in *Caenorhabditis elegans*

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To counteract replication-dependent telomere shortening most eukaryotic cells rely on the telomerase pathway, which is crucial for the maintenance of proliferative potential of germ and stem cell populations of multicellular organisms. Likewise, cancer cells usually engage the telomerase pathway for telomere maintenance to gain immortality. However, in ~10% of human cancers telomeres are maintained through telomerase-independent alternative lengthening of telomeres (ALT) pathways. Here, we describe the generation and characterization of *C. elegans* survivors in a strain lacking the catalytic subunit of telomerase and the nematode telomere-binding protein CeOB2. These clonal strains, some of which have been propagated for >180 generations, represent the first example of a multicellular organism with canonical telomeres that can survive without a functional telomerase pathway. The animals display the heterogeneous telomere length characteristic for ALT cells, contain single-stranded C-circles, a transcription profile pointing towards an adaptation to chronic stress and are therefore a unique and valuable tool to decipher the ALT mechanism.

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Introduction

With the exception of few organisms such as *Drosophila melanogaster* (Pardue *et al*, 1996), telomere maintenance in the germ line of multicellular life forms is achieved through the telomerase enzyme, a specialized reverse transcriptase that adds telomeric repeats to the ends of linear chromosomes (Blackburn, 1992; McEachern *et al*, 2000). Telomerase is highly active in stem cell populations and germ cells in most mammalian organisms, but is frequently downregulated in somatic tissues, which limits the replicative potential in these tissues and acts as tumour-suppressive mechanism (Shay and Wright, 1996; de Lange, 1998; Verdun and Karlseder, 2007; O'Sullivan and Karlseder, 2010). Consequently, loss of telomerase function through targeted deletion of the telomerase catalytic subunit or the telomerase RNA template in mice has initially no effect on the organism due to the existence of sufficient telomeric sequence (Blasco *et al*, 1997; Liu *et al*, 2000). However, due to replication-associated telomere shortening in these mutants, lack of functional telomerase eventually becomes detrimental to the whole organism, as it leads to telomere dysfunction in germ cells and proliferative organs and ultimately to telomere fusions, resulting in infertility, chromosomal aberrations and genomic instability (Blasco *et al*, 1997; Liu *et al*, 2000; Meier *et al*, 2006). Similarly, cancer cells have to circumvent replicative telomere shortening and engage pathways to extend telomeres in order to gain unlimited proliferation potential. In about 90% of cancers, this is achieved through activation and upregulation of the telomerase pathway (Shay and Wright, 1996; Verdun and Karlseder, 2007; O'Sullivan and Karlseder, 2010). However, in ~10% of cancers telomerase remains downregulated and telomeres are maintained through a telomerase-independent mechanism called alternative lengthening of telomeres (ALT) (Bryan *et al*, 1995, 1997). Telomere maintenance through ALT occurs through recombination-dependent pathways, although the precise mechanisms involved are still elusive (Dunham *et al*, 2000). Mounting evidence suggests that ALT can be activated upon the experimental suppression of telomerase, suggesting that it could be the dominant-negative pathway and emphasizing the need for understanding the ALT underlying molecular pathways.

In the nematode *C. elegans*, the gene *trt-1* has been identified as the catalytic subunit of telomerase (Meier *et al*, 2006). Accordingly, mutations in this gene have an effect on the organism comparably to the loss of telomerase activity in the mouse model. Initially, *trt-1* deletions do not affect the organism, but eventually progressive telomere erosion causes genomic instability resulting in sterility (Meier *et al*, 2006). To date, no survivor worms that overcome telomerase deficiency and compensate for *trt-1*-dependent telomere maintenance by activating alternative pathways have been isolated. This is surprising, since previous characterization of *C. elegans* telomere structure and telomere-binding proteins revealed features in the worm that are typical for ALT in mammalian cells. First, whereas the occurrence of 3′ single-stranded G-rich overhangs is a universal feature of most species described, *C. elegans* telomeres possess both 3′ G-rich single-stranded overhangs and 5′ C-rich single-stranded overhangs in equal amounts (Raices *et al*, 2008). Such 5′ C-rich telomeric overhangs have now also been reported in human cell lines and appear to be a characteristic of ALT. While their abundance is

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very low in primary human cells and telomerase-positive cancer cells, 5′ C-rich telomeric overhangs are highly abundant in cancer cells that use the ALT pathway for telomere maintenance (Oganesian and Karlseder, 2011). Second, a mutation in one of the *C. elegans* telomeric binding proteins results in an ALT-like phenotype: a loss-of-function mutation in the single-stranded telomeric C-strand binding protein CeOB2 (also known as POT-1; sequence name: B0280.10) results in increased telomere length heterogeneity with telomere length ranging from very short to extremely long, which is a feature usually found in ALT cells (Raices *et al*, 2008; Henson *et al*, 2009). Taken together, this suggested that *C. elegans* might have the capacity for ALT under circumstances that facilitate the activation of this pathway.

Here, we report the generation and characterization of a *C. elegans* strain that can propagate indefinitely in the absence of a functional telomerase pathway. We took advantage of the ALT-like properties in the *ceob2* mutant background to generate clonal *ceob2/trt-1* mutant strains, which can survive in the absence of telomerase. Some of the worm ALT strains have now been grown for >180 generations in the laboratory, representing the first example of a multicellular organism with canonical telomeres that can propagate despite an abrogated telomerase pathway.

Results and discussion

Generation of telomerase-negative survivor strains

To generate telomerase-negative survivor strains in *C. elegans*, we took advantage of the ALT-like telomere phenotype of a strain with a mutation in the telomere-binding protein CeOB2 (Raices *et al*, 2008). This strain exhibits increased telomere length heterogeneity reminiscent of human ALT cells (Raices *et al*, 2008). Single-stranded (ss) C-rich telomeric DNA is present in ALT$^+$ cells but not in telomerase-positive or mortal cells (Grudic *et al*, 2007). Most of the ss-C-rich DNA is likely extrachromosomal and circular and recently it has been suggested that partially single-stranded telomeric C-circles can be used as an accurate marker of ALT activity (Henson *et al*, 2009; Oganesian and Karlseder, 2011). C-circles can be rapidly and quantitatively detected by an assay based on rolling circle amplification using Φ29 DNA polymerase (Henson *et al*, 2009). To determine if *ceob2* mutant worms exhibited increased levels of C-circles, we modified the existing C-circle assay protocol for the use with *C. elegans* telomeric DNA and examined the levels of C-circles in wild-type (wt) N2 strains and *ceob2* mutant strains. Genomic DNA from a human ALT cell line (KMST-6) and a primary human fibroblast cell line (IMR90) served as controls. C-circles were almost undetectable in the primary human cell line, but massively increased in the ALT cell line (Figure 1A). A small amount of C-circles was detected in wt *C. elegans*, and C-circle levels were highly elevated in the *ceob2* mutant worms (Figure 1B), which indeed suggest increased telomeric recombination in this background. Dot blot analysis of C-circles that allow quantification of the polymerase products also revealed strongly increased C-circle formation in *ceob2* mutants (Figure 1D and E). The rolling circle amplification activity of Φ29 was assayed with M13 bacteriophage circular single-stranded DNA (Figure 1C).

We hypothesized that this increased telomeric recombination potential could prime *ceob2* mutants for ALT-like survival and could consequently allow for the isolation of telomerase-negative survivors that can maintain their telomeres through telomerase-independent mechanisms. To test this hypothesis, we set up crosses between early generation *trt-1* mutants and *ceob2* deletion mutants, isolated wt, single and double mutants (Figure 2A) and tested independent clones from each genotype for long-term survival. We grew the worms at 15°C to minimize genomic instability and we transferred 5–6 larvae of each genotype to a new feeding plate every two generations. As expected, telomerase-positive wt animals and the *ceob2* single mutants did not show a decline in fecundity even after multiple transfers. In contrast, all *trt-1* single mutant lines showed a gradual decrease in brood size and eventually became sterile (F16-26), as described previously (Meier *et al*, 2006) (Figure 2B and C, left panels). However, co-mutation of *ceob2* and *trt-1* allowed the isolation of several double mutant (DM) survivor clones that have been growing for over 180 generations (Figure 2D, left panel) and displayed mostly medium brood sizes. To assess the possibility that these worms had only survived due to the presence of long telomere background inherited from the *ceob2* strain, we crossed *trt-1* mutant worms with the unrelated wt strain AB2 that has very long telomeres (Raices *et al*, 2005). We analysed sterility onset in worms that were out-crossed once or four times with the AB2 strain and we observed that loss of fecundity was delayed as a function of telomere length. While *trt-1* mutants that were out-crossed to AB2 worms once became sterile between generations 14 and 36, *trt-1* worms that were out-crossed into the AB2 background four times displayed longer telomeres and could survive for up to 72 generations (Figure 3A–C). However, *trt-1* mutants in the AB2 background became sterile in all cases and survivors could never be isolated.

To corroborate that the occurrence of DM survivor strains was facilitated by the lack of CeOB2, we repeated the crosses and again we could isolate survivors from several *trt-1 ceob2* DM lines (Figure 2B–D, right panels). In this independent experiment, a single *trt-1* mutant line survived until generation 72, but eventually became sterile, suggesting that in some instances the variable clonal telomere length of individual animals (Raices *et al*, 2005) allows extended periods of survival without telomere maintenance. However, towards the end of the sterility assay this *trt-1* mutant line displayed strong developmental defects and had a very small brood size, whereas all wt controls and the DM survivors were still reproductively active. The loss of CeOB2 in *trt-1* mutant strains does not necessarily and immediately lead to an activation of ALT, illustrated by the fact that most clonal DM lines eventually become sterile. However, our data strongly suggest that the lack of functional CeOB2 in the survivor strains initially can prime these telomeres for maintenance mechanisms that can function in the absence of functional telomerase. Given the increased levels of C-circles in *ceob2* mutants, we suggest that this likely happens due to increased telomeric recombination events, which in certain instances can lead to the generation of survivor strains. We therefore also tested the levels of C-circles in two *trt-1 ceob2* DM lines (B, D). Both lines exhibited increased levels of C-circles, albeit to a different extent (Figure 4A and B), suggesting persistent recombination activity in these animals.

The survivor DM lines exhibited similarities to late generation *trt-1* mutants in several aspects. They exhibited reduced brood sizes, signs of genomic instability such as a

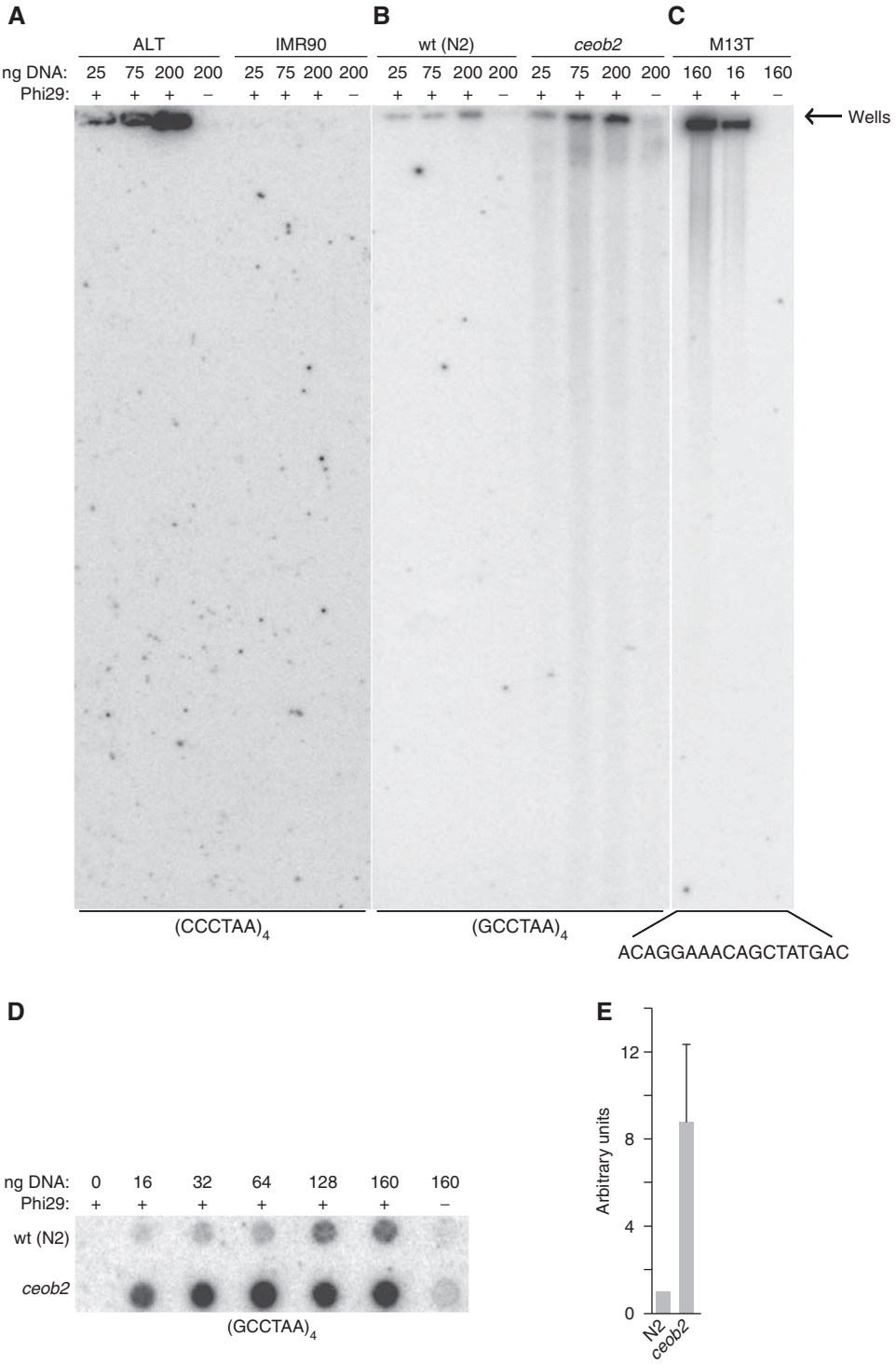

Figure 1 C-circles are elevated in *ceob2* mutant *C. elegans*. (**A**) C-circle assay using increasing concentrations (25, 75 and 200 ng) of genomic DNA from KMST-6 ALT cells and IMR90 fibroblasts. Long G-strand products migrated minimally from the wells. The presence (+) or absence (−) of Φ29 DNA polymerase has been indicated. Gels were hybridized using a $(CCCTAA)_4$-labelled probe. (**B**) C-circle assays using increasing concentrations (25, 75 and 200 ng) of genomic DNA from wild-type (N2) and ceob2 mutant worms. The presence (+) or absence (−) of Φ29 DNA polymerase has been indicated. Gels were hybridized using a $(GCCTAA)_4$-labelled probe. (**C**) M13 bacteriophage circular ssDNA (16 and 160 ng) was used as control template (M13T) for Φ29 rolling circle amplification activity. The presence (+) or absence (−) of Φ29 DNA polymerase has been indicated. (**D**) Dot blot of C-circle assay performed with serial dilutions of genomic DNA from wild-type (N2) and *ceob2* mutant worms with (+) and without (−) Φ29 DNA polymerase. Blots were hybridized using a $(GCCTAA)_4$-labelled probe. (**E**) Quantification of the C-circle amplification signals detected in *ceob2* mutants relative to wild-type N2 animals. The average of two independent experiments using five different concentrations of DNA as in (**D**) is shown.

high incidence of males (HIM) phenotype, developmental phenotypes such as larval arrest and vulva deformations (Table I) and altered karyotypes (see below for details). In other aspects the survivor lines were unique and different from *trt-1* mutants, since they exhibited variable brood sizes that did not consistently decline over time, relatively normal

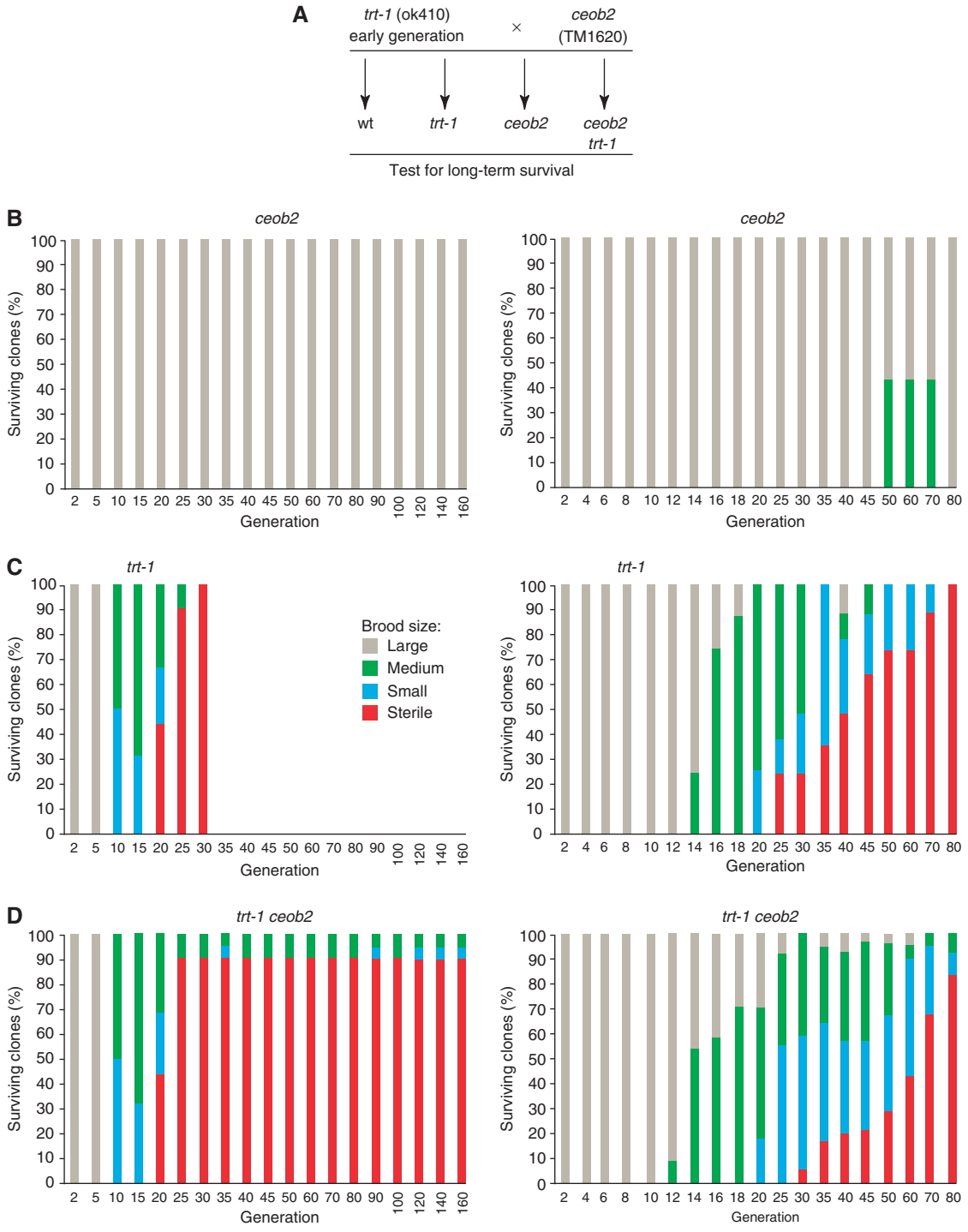

Figure 2 A trt-1/ceob2 double mutation supports indefinite propagation without telomerase. (**A**) Schematic of a cross between *trt-1* and *ceob2* mutant worms. (**B**) Brood size assay of *ceob2* mutant animals, (**C**) *trt-1* mutant animals and (**D**) *trt-1/ceob2* DM worms. Grey, green and blue bars represent wild-type, medium and small brood sizes, respectively. Red bars indicate sterility. The percentage of surviving clones has been plotted against generation number. The left and right panels represent two independent experiments.

levels of germ-cell proliferation and a large number of unhatched embryos, which could be observed on the bacterial lawns (Table I).

Heterogeneous telomere length of trt-1 mutant survivor strains

We next analysed the telomere length of the survivor strains using telomeric restriction fragment (TRF) analysis by probing *Alu*I/*Mbo*I-digested genomic DNA with telomere-specific probes allowing detection of telomeric repeats (Raices *et al*, 2008). Survivors from both independent crosses displayed strong heterogeneity in their telomere length (Figure 4C). While the bulk of telomeric signal stemmed from very short telomeres, several high-molecular weight bands could be detected. These bands were even more prominent in clonal survivors from the second cross, where they were reminis-

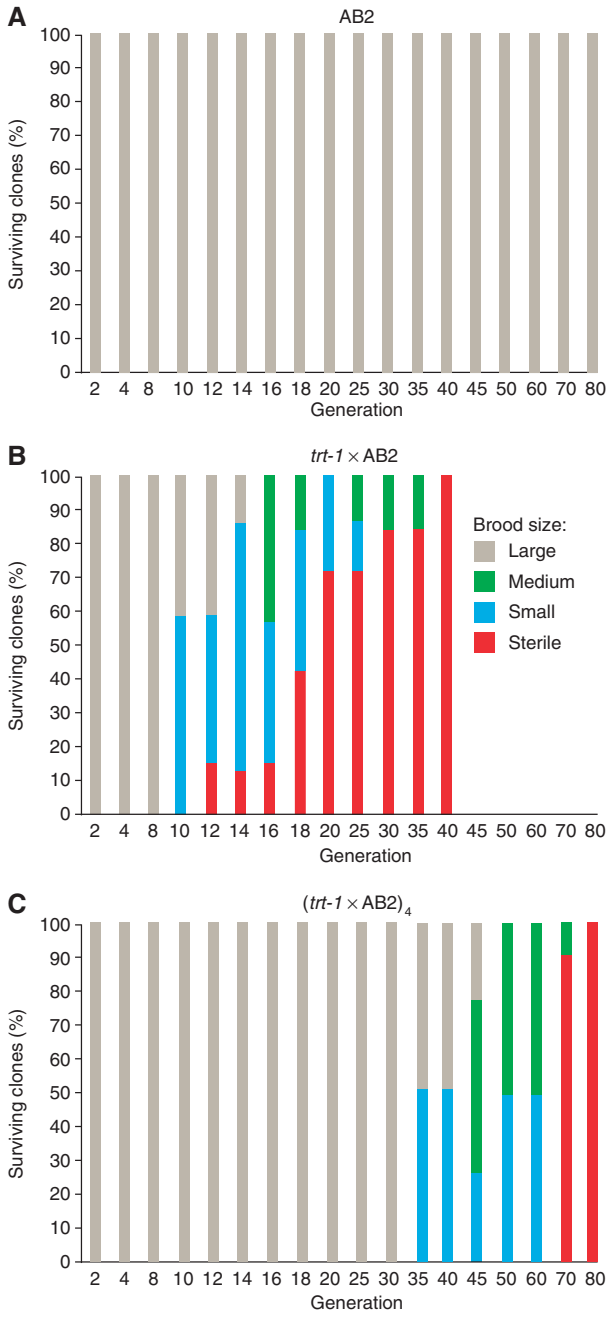

Figure 3 Longer telomeres allow for longer survival without telomerase. (**A**) Brood size of AB2 animals over 80 generations and (**B**) *trt-1*/AB2 animals that have been out-crossed once into the AB2 strain and (**C**) *trt-1*/AB2 animals that have been out-crossed four times into the AB2 strain. Grey represents wild-type brood sizes, green medium, blue small brood sizes and red indicates sterility. The percentage of the original clones still surviving has been plotted against generation number.

cent of the population of heterogeneous telomeres in the *ceob2* deletion mutants (Figure 4C, right panel). We also followed the telomere length dynamics of one DM survivor strain (DM B, now grown for over 180 generations), for 80 generations (Figure 4D). While the telomeres of a wt control or the *ceob2* mutant strain remained constant in length, the size of the high-molecular weight bands in the survivor strain changed frequently. This heterogeneous nature of telomere length in the survivors is similar to human ALT cells and suggests increased recombination between short and long telomeres.

Chromosome fusions in survivor strains

The appearance of many distinct, higher molecular weight heterogeneous bands in the TRFs of the survivor strains suggests that some of them might stem from telomeric fusions. To test the strains for fusions, we assayed chromosome numbers by staining young adult worms with DAPI and counting the number of chromosomes in the oocytes, which are the only metaphase cells in worms, supporting visualization of individual chromosomes (Figure 5A). Oocytes from a wt strain isolated from a *trt-1* and *ceob2* mutant cross mostly contained six chromosomes (Figure 5A and B). In a few cases, only five chromosomes were observed, most likely due to two chromosomes being in close proximity and the lack of resolution of the assay. In contrast, in two survivor strains tested (DM B strain from first cross and DM D strain from second cross) we largely observed less than six chromosomes (Figure 5A and B), indicating that these strains survive with fused chromosomes. The number of chromosomes was not random in the survivor strains. In the DM B strain, we observed three chromosomes (67% of oocytes); and in the DM D strain, we observed five chromosomes (77% of oocytes) (Figure 5B).

To further corroborate the existence of telomeric chromosome fusions, we digested genomic DNA with the exonuclease Bal-31 prior to restriction digestion with *Alu*I/*Mbo*I and TRF analysis. Bal-31 degrades both 3′ and 5′ termini of duplex DNA, thus, telomeric DNA at chromosome ends is degraded, whereas internal telomeric repeats are kept intact during a limited digestion. Bal-31 treatment of wt genomic DNA reduced the telomeric signal during a 10-min digest and most of the hybridization signal was completely removed after a 90-min digest (Figure 5C, left panel), while some signals in the DMs were more resistant to Bal-31, indicating longer telomeres (Figure 5C, right panels). Also, several high-molecular weight bands from two DM survivor strains were fully resistant to Bal-31 digestion, indicating that these bands represent signals from internal telomeric repeat DNA as a consequence of telomere–telomere fusions.

We noted that the signal intensity of telomeric restriction fragments of the high-molecular weight material in the DMs was less intense than the signal in the CeOB2 lanes (Figure 4C and D). This could simply be due to the fact that the very long CeOB2 telomeres result in a single signal, compared with the many signals in the DMs. Alternatively, it could suggest that the signals represented fusions of telomeres with short telomeric tracts, and a potential interplay between telomere length, fusion formation and recombination activity.

Taken together, these data suggest that the survivor strains live with semistable chromosomal aberrations that allow adaptation to the detrimental consequences of genomic instability in response to telomere erosion.

Similar gene-expression profiles in survivor strains and late generation trt-1 mutants

To investigate whether this adaptation to stress in the survivors is reflected in global gene-expression profile changes, we compared early and late generation *trt-1* mutants with DM survivors. RNA was prepared from the DM B strain and corresponding wt (wt F110) and *ceob2* deletion strains gen-

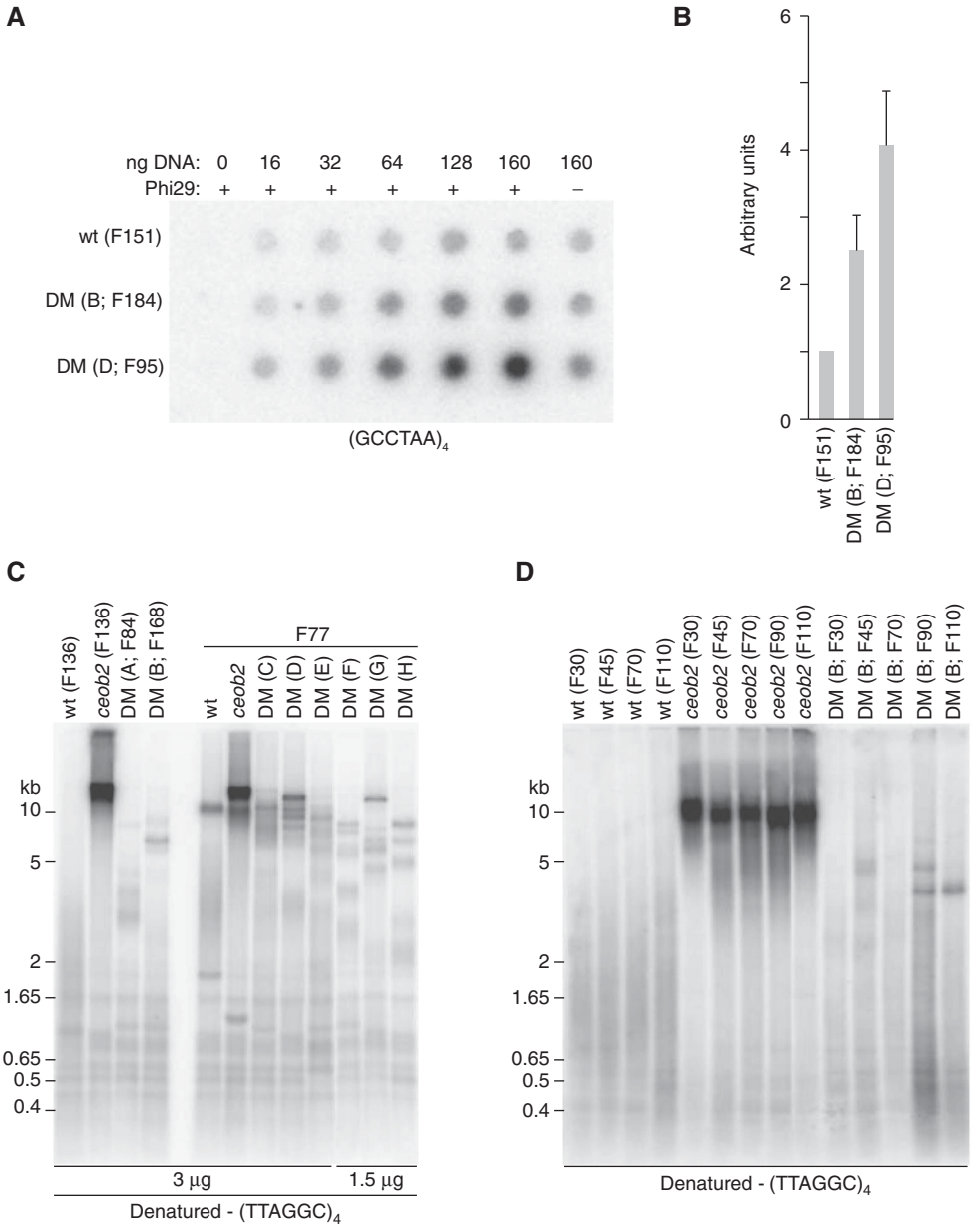

Figure 4 *trt-1/ceob2* double mutants have increased C-circles and heterogeneous telomeres. (**A**) Dot blot of C-circle assay performed with serial dilutions of genomic DNA from wild-type (wt) and *trt-1/ceob2* double mutant (DM) animals with (+) and without (−) Φ29 DNA polymerase. Blots were hybridized using a $(GCCTAA)_4$-labelled probe. (**B**) Quantification of the C-circle amplification signals detected in *trt-1/ceob2* double mutant (DM) animals relative to wild-type (wt) animals. The average of two independent experiments using five different concentrations of DNA as in (**A**) is shown. (**C**) Telomere length analysis of wild-type (wt), *ceob2* mutant (*ceob2*) and *trt-1/ceob2* double mutant (DM) animals from two independent experiments. Two strains from the first cross (left panel) and six strains from the second cross (right panel) were analysed. DNA size is indicated on the left and amount loaded on the bottom. (**D**) Telomere length of wild-type (wt), *ceob2* mutant (*ceob2*) and *trt-1/ceob2* double mutant (DM) animals over 80 generations. DNA size is indicated on the left and 3 µg of DNA was loaded per lane.

Table I Phenotypes of mutant animals

Phenotype	ceob2	trt-1	trt-1/ceob2
Small brood size	N	Y	Y
HIM	N	Y	Y
Larval arrest	N	Y	Y
Vulva defects	N	Y	Y
Empty gonad arms	N	Y	Y
Cell proliferation in gonads	Y	N	Y
Embryos in gonads	Y	Few	Y
Unhatched embryos	N	Few	Y

erated from the first cross, which had been grown for 110 generations. Additionally, we prepared RNA from wt animals as well as from early (F3, F7) and late (F11) generation *trt-1* mutant animals, generated from backcrossed heterozygous *trt-1* mutants. As expected, telomeres shorten over generations in the homozygous *trt-1* mutant strains but stay stable in the wt control strains (Supplementary Figure S1). Expression profiles of early generation *trt-1* mutant strains and the *ceob2* mutant strain were remarkably similar to wt animals, only showing 1 gene differentially expressed in early

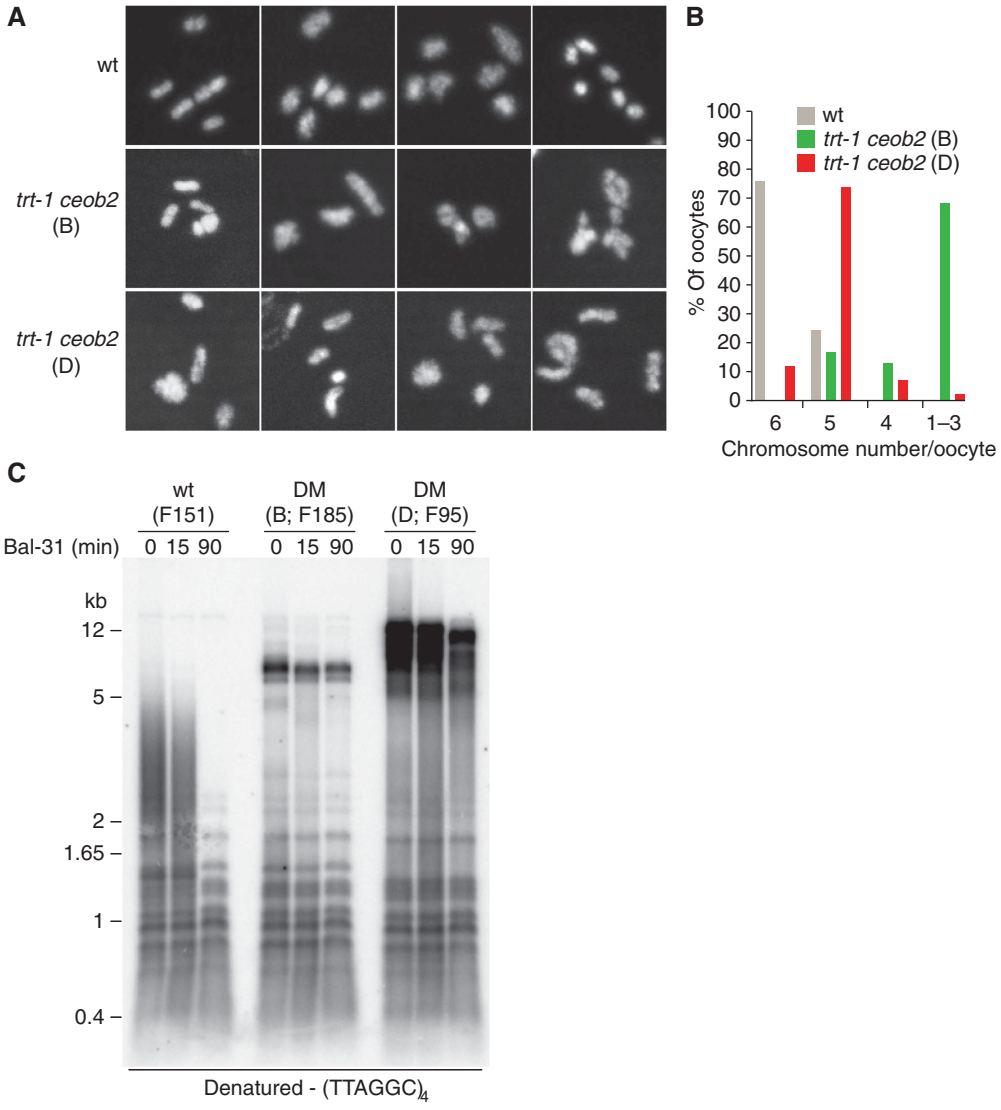

Figure 5 Survivor strains contain fused chromosomes. (**A**) DAPI staining of mitotic chromosomes in oocytes in the nematode germ line. Each panel represents chromosomes from one oocyte. (**B**) Quantification of chromosome numbers in wild-type (wt) animals ($n = 63$) and two *trt-1/ceob2* DM survivor strains (DM B ($n = 37$) and DM D ($n = 82$)). (**C**) Telomere length analysis of wild-type (wt), and *trt-1/ceob2* double mutant (DM) animals after incubation of genomic DNA with Bal-31 nuclease for the indicated time. DNA size is indicated on the left.

generation *trt-1* (F3), 3 genes differentially expressed in F7 *trt-1* mutants and 24 genes differentially expressed in the *ceob2* mutant worms (Supplementary Table 1). We suggest that these changes reflect subtle strain-specific differences or experimental noise, since we observed more significant changes between the two different wt controls (43 genes changed) (Supplementary Table 1).

In contrast, we could observe multiple changes in the transcriptome in late generation *trt-1* mutants and the DM survivor strain, with 834 genes differentially expressed in the late generation *trt-1* mutant strain and 2370 genes differentially expressed in the survivor strain (Supplementary Table 1). These data suggest that mutation of *trt-1* has no immediate effects on *C. elegans*, but that large gene-expression changes arise in response to extensive telomere erosion and DNA damage. This is consistent with the reported gene-expression changes in mice with a deletion of the telomerase gene, where increased gene-expression changes are observed in later generation knockout mice and are associated with increasing DNA damage due to loss of telomeric sequence buffer (Vidal-Cardenas and Greider, 2010; Sahin *et al*, 2011). The connection between loss of telomeric sequence and increasing gene-expression changes was also confirmed focusing on the expression of an individual gene that is expressed from the telomeric region of the X chromosome: cTel55X.1 shows a strong downregulation only in the late generation *trt-1* mutant as well as in the survivor strain (Figure 6A). This downregulation of cTel55X.1 is not a consequence of physical loss of the gene due to telomere erosion, which was confirmed by PCR from genomic DNA across the cTel55X.1 locus (Supplementary Figure S3). To further analyse transcriptome similarities between *trt-1* mutants and the survivor strain, we applied hierarchical clustering (Figure 6B). The late generation *trt-1* mutant and the survivor strain clustered closely together, whereas the early generation *trt-1* mutant strains, the *ceob2* mutant strain and

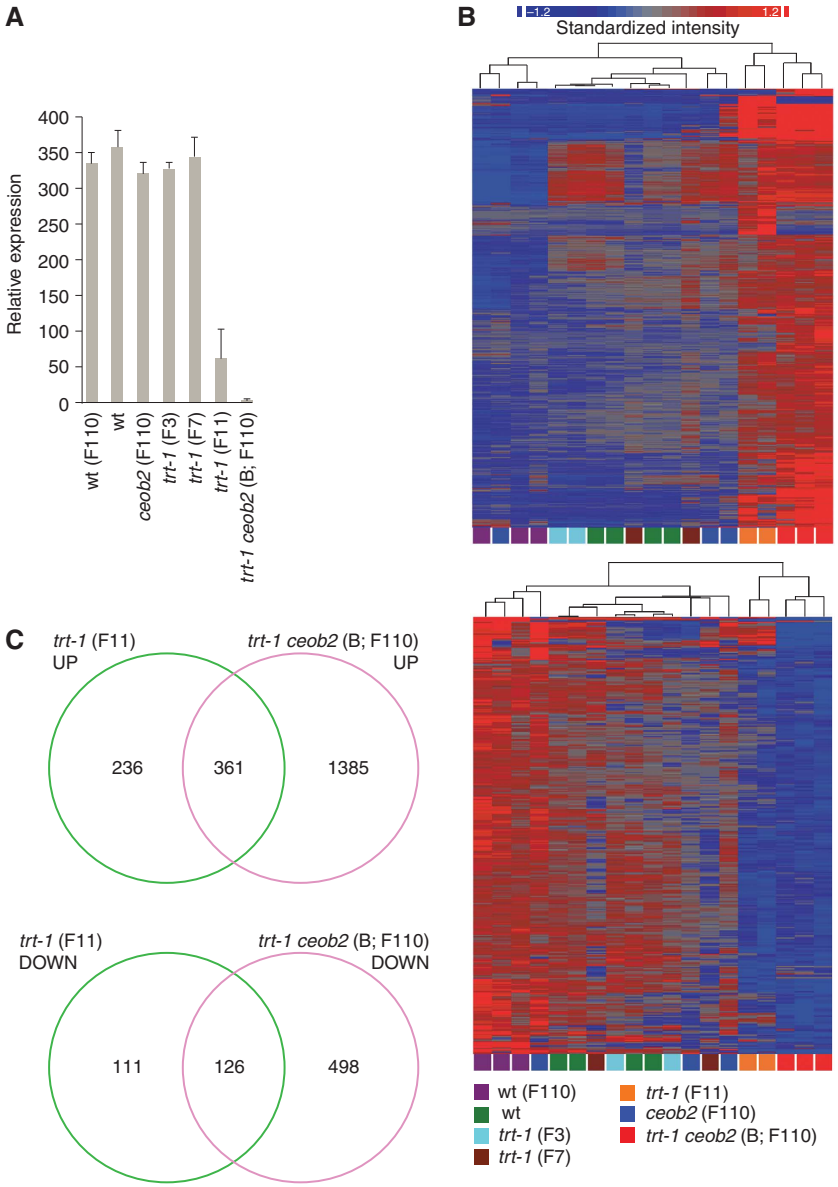

Figure 6 Similar gene-expression profiles between the survivor strain and late generation *trt-1* mutants. (**A**) Relative expression levels of CTel55X.1, a gene expressed from the telomeric region of the X chromosome. (**B**) Hierarchical clustering of genes regulated two-fold in either late generation *trt-1* mutant or a DM survivor strain. The upper panel shows clustering of 1982 upregulated genes and the lower panel shows a cluster of 735 downregulated genes. Genotypes corresponding to the individual Affymetrix arrays are indicated at the bottom. Expression levels were standardized by shifting the mean to zero. (**C**) Venn diagrams depict the overlap of genes regulated two-fold in either late generation *trt-1* mutants or a DM survivor strain as depicted in (**B**).

the wt control animals clustered only randomly. There was also significant overlap between the regulated genes in the late generation *trt-1* mutant and the survivor strain (Figure 6C) and this trend extended to genes that did not meet the cutoff criteria (see Materials and methods) (Figure 6B and C). Genes that were two-fold upregulated only in the late generation *trt-1* mutants were on average also 1.2-fold upregulated in the DM survivor, and genes that were two-fold upregulated only in the DM showed on average an 1.5-fold upregulation in the late generation *trt-1* mutants. The same trend applied for downregulated genes: genes that were two-fold downregulated only in the late generation *trt-1* mutants were on average also 1.7-fold downregulated in the DM survivor, and genes that were two-fold downregulated only in the DM showed on average an 1.3-fold downregulation in the late generation *trt-1* mutants. Only one gene (*clec-190*) exhibited a completely contrary behaviour and was two-fold upregulated in the late generation *trt-1* mutants but two-fold downregulated in the DM survivor. This is likely due to a general variable gene expression of this gene, as it is also differentially regulated (2.6-fold) between the two different wt controls. We next analysed the regulated genes for enriched functional groups (Supplementary Table 2). Downregulated genes showed an enrichment of gene ontology (GO) terms related to general transcription (nucleic acid binding, DNA binding, transcription factor activity), which simply might reflect a systemic problem. The most enriched GO terms within upregulated genes were associated with

structural components of the animal (structural constituent of cuticle, structural molecule activity). From this analysis, we could not observe an obvious connection to processes related to DNA damage or genomic instability. However, when we compared upregulated genes in the DM survivor strain with previously reported IR-induced DNA-damage responsive genes (Greiss *et al*, 2008) we found that many genes overlapped. This suggests that this strain can survive in the presence of constant DNA-damage signalling (Supplementary Figure S2; Supplementary Table 3). However, downregulated genes in the survivor strain showed almost no overlap with genes downregulated in response to IR-induced DNA damage (Supplementary Figure S2).

We next tested, if other DM survivor lines had a similar expression profile to the DM survivor line B. Expression profiling for the DM C and DM D survivor lines and a wt control revealed a slightly lesser extent of regulation in general, as the number of regulated genes was lower in the DM C and DM D lines compared with wt animals (243 and 447 genes upregulated, 34 and 60 downregulated, respectively). However, the general trend of regulation was similar to the DM B survivor lines, as pointed out by the hierarchical clustering of genes regulated in the DM B survivor according to the gene-expression values in the DM C and DM D survivor lines (Supplementary Figure S4). Genes that are two-fold upregulated in the DM B line also displayed an average upregulation of 1.5 and 1.7 in the DM C and DM D lines, respectively. Genes that are two-fold downregulated in the DM B line also showed an average downregulation of 1.2 and 1.3 in the DM C and DM D lines. Some difference in gene-expression profile between different DM survivor lines is not surprising, given that they acquire different sets of chromosomal aberrations and mutations due to genomic instability. However, an overall similar trend of regulated genes could be observed within the different DM survivor strains, which is directly reflected in the strong similarity of enriched functional groups within the regulated genes for each DM strain (Supplementary Table 2).

Conclusion

The *C. elegans* strains presented here are the first example of a multicellular organism with canonical telomeres that can propagate indefinitely through a telomerase-independent ALT-like pathway after mutation of the catalytic subunit of telomerase. Clonal survivors exhibit signs of genomic instability and a gene-expression profile similar to the late generation *trt-1* mutants, but manage to adapt to the chronic DNA damage and thrive, suggesting that telomerase loss or critically short telomeres predispose cells and organisms to activate ALT pathways. Survival is primed by a loss-of-function mutation of CeOB2, which renders telomeres heterogeneous, similarly to human ALT cells. We suggest that telomeres in this setting are more recombinogenic and thus are primed for maintenance through an ALT-like pathway. Additionally, the holocentric nature of *C. elegans* chromosomes might render them less susceptible to detrimental chromosome fusion-breakage-fusion cycles (Dernburg, 2001). We propose that these survivor strains could represent a unique and valuable tool to study ALT in an organismal context and to aid in deciphering the underlying molecular mechanisms of ALT.

Materials and methods

Strains and sterility assays

Early generation *trt-1* mutant (ok410) hermaphrodites were crossed with males generated from the *ceob2* deletion strain (tm1620). Single F1 heterozygous worms were used to generate *trt-1*, *ceob2* and *trt-1/ceob2* mutant genotypes. Since clonal differences in telomere length occur in *C. elegans* (Raices *et al*, 2005) all the lines analysed in each experiment came from the same heterozygous parent to ensure that worms had the same telomeric background. For the first cross, 12 lines of each genotype were isolated. Brood size was determined by examining plates seeded with 5–6 L1 larvae after growing them for two generations at 15°C on small NG plates. Plates were scored as wt, medium, few and sterile depending on the amount of progeny. Initially, 10 plates for each individual line were scored. Independent plates from the same line showed similar amounts of progeny. As *trt-1* mutants approached sterility we noticed that two of the *trt-1/ceob2* mutant lines displayed high variability in the amount of progeny having plates that looked like *trt-1* mutants and others with larger brood sizes. While some of the plates from these lines eventually became sterile other plates stayed reproductive (survivors). Survivor worms displayed variable brood sizes over generations and were grown for 170 generations on both small and large plates. The second cross was done in the same way, but with fewer lines: for the *ceob2* mutant, we analysed three plates each of three independent lines; for the *trt-1* mutant, we analysed eight plates of one line; and for the *trt-1/ceob2* mutant, we analysed eight plates each of three different lines.

Telomere length analysis

Genomic DNA preparation, TRF analysis and Bal-31 digestions were done as described previously (Raices *et al*, 2005, 2008).

C-circle assays

The method for the assay probing for C-circles was modified for *C. elegans* and adapted from Henson *et al* (2009). Worm genomic DNA was purified and digested with *Alu*I and *Mbo*I as described (Raices *et al*, 2008). Samples (10 μl) were combined with 10 μl 0.2 mg/ml BSA, 0.1% Tween, 1 mM each dATP, dGTP, dTTP, dCTP and 1 × Φ29 Buffer in the presence or absence of 7.5 U ΦDNA polymerase (NEB). Samples were incubated at 30°C for 8 h and then at 65°C for 20 min. Reaction products were separated in 0.6% agarose gels using 0.5 × Tris-borate-EDTA at 1.75 V/cm for 12 h. Gels were dried at 50°C and hybridized under native conditions as described (Raices *et al*, 2008). Alternatively, samples were diluted to 60 μl with 2 × SSC and dot-blotted onto a 2 × SSC-soaked nylon membrane. DNA was UV crosslinked onto the membrane and hybridized as described. The $(GCCTAA)_4$ probe was used for detecting C-circle amplification products.

M13mp18ss circular DNA (NEB) was used as a control template for Φ29 activity as described (Henson *et al*, 2009). Genomic DNA from ALT (KMST-6) cells was used as a positive control for C-circle detection and genomic DNA from IMR90 primary fibroblasts was used as a negative control. In these cases, amplification products were detected using the $(CCCTAA)_4$-labelled probe. All blots were exposed to PhosphoImager screens, scanned and quantified using a Typhoon 9400 PhosphoImager (Amersham/GE Healthcare).

DAPI staining

Bacteria were removed by washing worms three times in 1 ml of M9 medium in a watch glass. DAPI (200 μl; 200 ng/ml in ethanol) was added and incubated for 20 min. Samples were rehydrated in 1 ml of M9 for 1 h and then transferred to coverslips and mounted with ProLong Gold antifade reagent (Invitrogen). Images were taken on a Zeiss Axio Imager Z1 at × 63 magnification. Z-stacks were obtained using the ApoTome and images were displayed using maximum projection to count the number of chromosomes in oocytes.

Expression profiling and statistical analysis

For each condition, RNA was extracted from synchronized young adults from at least two independent biological replicates. Worms were washed with M9 medium and the worm pellet was resuspended in 1 ml of Trizol (Invitrogen) and subjected to four

freeze-thaw cycles in liquid nitrogen. RNA was extracted according to manufacturer's instructions and afterwards purified using RNeasy mini kits (Qiagen). Isolated RNA (100 ng) was used for generation of cDNA and terminal labelling using the 3′ IVT Express Kit (Affymetrix) and labelled probes were hybridized to GeneChip *C. elegans* Genome Arrays (http://www.affymetrix.com/estore/browse/products.jsp?productId = 131405&categoryId = 35878&productName = GeneChip-C.-elegans-Genome-Array) representing 22 500 transcripts. Arrays were scanned on a GeneChip Scanner 3000 7G. Cell files were normalized using the Partek Genomics Suite (RMA background correction, quantile normalization, median polishing). Differentially expressed genes were detected using the ANOVA function in the Partek Genomics Suite. To define differentially expressed genes, we used a two-fold cutoff and a *P*-value adjusted for false discovery rate of <0.05. Hierarchical clustering was performed using the Partek Genomics Suite and expression values for the clusters were standardized by shifting the mean to zero. Enriched GO groups were defined using the Generanker tool in the Genomatix Genome Analyzer software (http://www.genomatix.de/en/produkte/genomatix-genome-analyzer.html).

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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