

# Requirement of functional telomeres for metaphase chromosome alignments and integrity of meiotic spindles

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**Telomerase deficiency in the mouse eventually leads to loss of telomeric repeats from chromosome ends and to end-to-end chromosome fusions, which result in defects in highly proliferative tissues. We show that telomere dysfunction resulting from telomerase deficiency leads to disruption of functional meiotic spindles and misalignment of chromosomes during meiotic division of oocytes in late-generation ( $G_4$ ) mice. However, oocytes from first-generation ( $G_1$ ) mice lacking telomerase showed no appreciable telomere dysfunction and exhibited chromosome alignment at the metaphase plates of meiotic spindles, in a manner similar to that of wild-type mouse oocytes. These findings suggest that telomerase does not directly influence chromosome alignment and spindle integrity. Rather, functional telomeres may be involved in mediating metaphase chromosome alignment and maintaining functional spindles during meiotic division.**

## INTRODUCTION

Vertebrate telomeres consist of tandem repeats of the sequence TTAGGG and of associated proteins, which cap the ends of chromosomes and protect them from degradation and fusion (Blackburn, 2001). Extensive evidence has shown that telomere shortening and dysfunction in cultured somatic cells leads to the so-called replicative senescence (Blackburn, 2000). In turn, reversal of telomere shortening by the forced expression of telomerase rescues cells from senescence and extends cell lifespan indefinitely (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998). Active telomerase, which is composed of a small RNA molecule, known as the telomerase RNA (TR), and of a catalytic subunit, the telomerase reverse transcriptase, is the primary enzyme for maintaining the length of telomere repeats.  $TR^{-/-}$  mice lack

telomerase activity and show progressive telomere shortening with increasing mouse generations, eventually resulting in telomere-exhausted chromosomes and chromosomal end-to-end fusions (Blasco *et al.*, 1997; Lee *et al.*, 1998; Herrera *et al.*, 1999a,b; Rudolph *et al.*, 1999; Gonzalez-Suarez *et al.*, 2000). Telomere dysfunction in late-generation  $TR^{-/-}$  mice leads to various pathologies, including defects in development, growth and immune function, and influences tumorigenesis. Female fertility also decreases with increasing  $TR^{-/-}$  mouse generations, as evidenced by reduction in litter size and compromised embryo development, eventually resulting in sterility (Lee *et al.*, 1998; Herrera *et al.*, 1999b).

Embryo development in mammals is initiated by fertilization of mature oocytes at the metaphase II (MII) stage of meiosis. In females, germ cells, or oocytes, are produced by a specialized cell division, called meiosis. Compared with mitosis and male meiosis, female meiosis is unique and characterized by two distinct phases: one characterized by a prolonged arrest in prophase I (PI) of meiosis and the other by meiotic resumption and division or maturation. In contrast to male germ cells, female germ cells enter meiosis and arrest at the diplotene stage of PI during fetal development and remain arrested at this stage, also called the germinal vesicle (GV) stage, for weeks in mice and years in humans until puberty, when the meiotic arrest is lifted by gonadotrophins. Gonadotrophin stimulations induce meiotic resumption and division of competent oocytes, as indicated by germinal vesicle breakdown, and progress through metaphase I (MI), anaphase I (AI) and telophase I (TI), until MII, where they arrest again until fertilization.

Pairing and genetic recombination of homologous chromosomes, unique to meiosis, occurs at leptotene/zygotene stages during the first meiotic prophase. Telomere clustering at the nuclear

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**Table I.** Meiotic progression of wild-type (WT) and TR<sup>-/-</sup> G<sub>4</sub> mouse oocytes<sup>a</sup>

Oocyte	9 h of culture			12–14 h of culture					
	Number examined	Stage of meiosis <sup>b</sup>			Number examined	Stage of meiosis <sup>b</sup>			
		GV–GVBD	MI (%)	AI–TI		GV–GVBD	MI	AI–TI	MII (%)
WT	39	1	31 (79%) 1 misaligned	7	28	0	3	0	25 (89%) 0 misaligned
TR <sup>-/-</sup> G <sub>4</sub>	17	6	6 (35%) 4 misaligned	5	21	5	3	1	12 (57%) 8 misaligned

<sup>a</sup>Determined by immunofluorescence staining and confocal microscope.

<sup>b</sup>GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase of meiosis I; AI, anaphase I; TI, telophase I; MII, metaphase of meiosis II.

envelope and the bouquet formation are thought to be prerequisite steps for the pairing and recombination of homologous chromosomes before the meiotic arrest (Scherthan *et al.*, 1996; de Lange, 1998; Tease and Fisher, 1998; Bass *et al.*, 2000; Scherthan *et al.*, 2000), although this has not been formally demonstrated in mammalian female germ cells. Meiotic chromosomes frequently missegregate in human females, leading to aneuploidies and a high frequency of failed implantation and miscarriages (Hassold *et al.*, 1996). Experiments in mice indicate that checkpoints for meiotic chromosome behavior at metaphase–anaphase transition are less efficient in females than in males (Hunt *et al.*, 1995; LeMaire-Adkins *et al.*, 1997). Despite the extensive interest in the function of telomeres in meiosis, there is no information on whether telomeres play a role in spindle organization and chromosome behavior during meiotic division in general and in mammalian female germ cells in particular.

By taking the advantage of telomerase knockout mice available, we sought to investigate whether telomerase deficiency and/or telomere dysfunction influence meiotic progressions by examining meiotic resumption and division (maturation) of GV oocytes from fourth-generation (G<sub>4</sub>) TR<sup>-/-</sup> mice (*n* = 8) and first-generation (G<sub>1</sub>) TR<sup>-/-</sup> mice, compared with that of wild type as controls. We found chromosome misalignment and disruption of meiotic spindles at metaphase stages in oocytes from G<sub>4</sub> TR<sup>-/-</sup> mice. However, meiotic progression, chromosome behavior and spindles in G<sub>1</sub> TR<sup>-/-</sup> mouse oocytes appeared to be comparable with those of wild-type mouse oocytes.

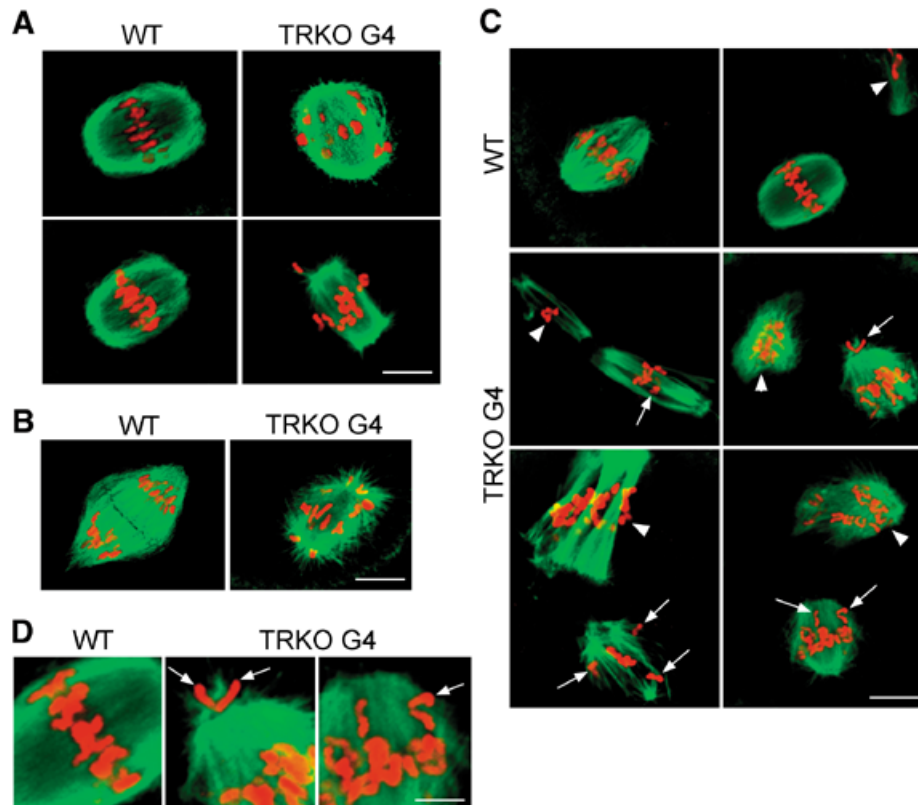
## RESULTS AND DISCUSSION

On average, 36 oocyte–cumulus complexes were collected from antral follicles of wild-type mice (*n* = 3), but only an average of 15 oocytes were obtained from G<sub>4</sub> TR<sup>-/-</sup> mice (*n* = 3). At MI stage, chromosomes from wild-type mouse oocytes were aligned on the metaphase plate of meiotic spindles, whereas over 50% of oocytes collected from G<sub>4</sub> TR<sup>-/-</sup> mice displayed abnormal meiotic I spindles and misalignment of chromosomes on the metaphase plate to different extents (Table I; Figure 1A). At the AI–TI stage, oocytes from wild-type mouse showed clear separation of chromosomes, with elongation of spindles. However, misaligned chromosomes were observed along the elongated and abnormal spindles from G<sub>4</sub> TR<sup>-/-</sup> mouse oocytes (Figure 1B). These abnormalities presumably could lead to chromosome missegregation and aneuploidy. Whereas all wild-type MII oocytes showed equatorial alignment of chromosomes in the middle of barrel-shaped bipolar spindles at metaphase,

G<sub>4</sub> TR<sup>-/-</sup> mouse MII oocytes (67%, 8 of 12) manifested abnormal chromosome alignments along the spindles (Table I; Figure 1C). Most oocytes exhibiting chromosome misalignments also showed disruption of spindles, some with unusual elongation and others with monopolar structures. In two G<sub>4</sub> TR<sup>-/-</sup> oocytes, which showed normal-appearing but shorter bipolar spindles, some chromosomes were not aligned on the metaphase plate (Figure 1C, bottom right); in others, the chromosome alignments were completely disordered and very few chromosomes were distributed on metaphase spindles and most were segregated into the first polar body extruded from their oocytes (Figure 1C, bottom left). Under higher magnification, one or more chromosomes were separated from the bulk of other chromosomes, and free chromosome ends were not connected to microtubules (Figure 1D). The high incidence of these lagging chromosomes coincided with the absence of chromosome end–microtubule attachment during anaphase proceeded in oocytes of G<sub>4</sub> mice lacking telomerase.

Progression from MI to MII stage occurs despite errors in the chromosome alignments at MI, further confirming that mammalian female meiosis lacks an efficient metaphase checkpoint control (LeMaire-Adkins *et al.*, 1997; Roeder, 1997), in contrast to mitotic cells (Taylor, 1999; Dobles *et al.*, 2000). The spindle checkpoint arrests cells in mitosis in response to defects in the assembly of the mitotic spindle or errors in chromosome alignment (Scolnick and Halazonetis, 2000). Many other chromosome- or kinetochore-associated proteins, such as Mps1, Mad2 and Xkid, have been shown to play a role in chromosome alignment at the metaphase plate of spindles (Antonio *et al.*, 2000; Dobles *et al.*, 2000; Funabiki and Murray, 2000; Abrieu *et al.*, 2001). Mutations in the telomeric DNA sequence of the ciliated protozoan *Tetrahymena thermophila* arrested nuclear division (Kirk *et al.*, 1997). The fragile meiotic checkpoint may be one of the factors related to ageing-associated infertility in humans. The defects in meiotic division resulting from telomere dysfunction could lead to aneuploidy and compromise subsequent embryo development, such as decreased rate of blastocyst development or increased embryonic mortality due to a neural tube closure defect (Lee *et al.*, 1998; Herrera *et al.*, 1999a). Hypoploidization of activated oocytes has been shown to result in apoptosis in embryos, preventing blastocyst formation (Liu *et al.*, 2002).

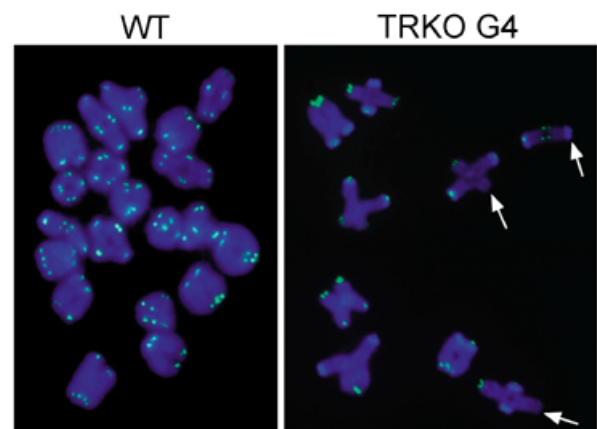
To detect the presence of TTAGGG repeats at the chromosome ends, we performed telomeric fluorescence *in situ* hybridization (FISH) on oocyte metaphase spreads using an FITC-labeled (CCCTAA)<sub>3</sub> peptide nucleic acid (PNA) probe, which is able to detect ≥200 bp of TTAGGG repeats at the telomeres. In somatic



**Fig. 1.** Chromosome alignment and spindles during meiotic division in wild-type (WT) and  $G_4$   $TR^{-}$  (TRKO) mouse oocytes. (A) MI stage at 9 h of culture, showing misalignment of chromosomes (red) on spindles (green) in  $TR^{-}$  mouse oocytes. (B) AI–TI stage, showing complete separation of chromosomes in wild-type mouse oocytes but chromosome scattering in  $TR^{-}$  mouse oocytes. (C) MII stage at 12–14 h of culture, showing misalignment (arrows) of chromosome over disrupted spindles in  $TR^{-}$  mouse oocytes, in contrast to equatorial chromosome alignment over the bipolar barrel-shaped spindles of wild-type mouse oocytes. Arrowheads indicate the first polar body. Scale bars (A–C), 10  $\mu$ m. (D) Higher magnification of (C), showing that aligned chromosomes appeared to attach to microtubules, whereas misaligned chromosomes were not attached but with free ends (arrows). Scale bar, 4  $\mu$ m.

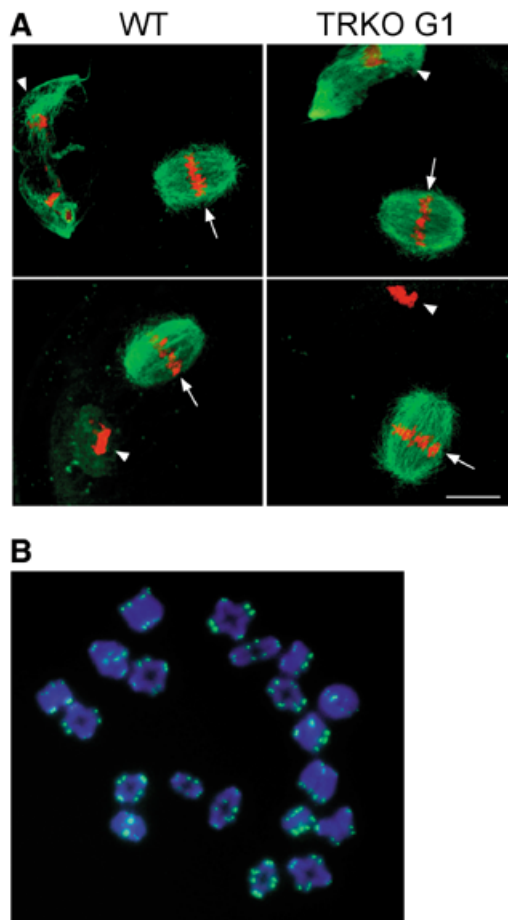
cells from late-generation  $TR^{-}$  mice, progressive telomere shortening leads to loss of FISH fluorescence signal from some chromosome ends and to chromosomal end-to-end fusions (Blasco *et al.*, 1997). Consistent with the observations made in  $TR^{-}$  male germ cells (Hemann *et al.*, 2001), we also could not find chromosome fusions in late-generation  $TR^{-}$  female germ cells. Accordingly, we used the absence of a telomere fluorescence signal on a chromosome end as an indicator of telomere dysfunction (Blasco *et al.*, 1997; Hande *et al.*, 1999; Hemann *et al.*, 2001). Telomere fluorescence signals were observed at almost all chromosome ends in the wild-type oocytes examined ( $n = 10$ ) at both MI and MII stages but were undetectable from 9.3% of the telomeres (37 chromosome ends lacking TTAGGG repeats from a total of 396 chromosome ends) in the chromosomes of  $G_4$   $TR^{-}$  mouse oocytes ( $n = 4$ , Figure 2), demonstrating that telomerase deficiency results in telomere erosion and dysfunction of  $G_4$   $TR^{-}$  oocytes.

The  $G_1$   $TR^{-}$  mice showed no obvious changes in telomere length and appeared to be normal in development, growth and fertility (Blasco *et al.*, 1997; Lee *et al.*, 1998; Herrera *et al.*, 1999a,b; Rudolph *et al.*, 1999). To determine whether lack of telomerase was responsible for defects observed in meiosis, we collected GV oocytes from  $G_1$   $TR^{-}$  mice and compared the behaviors of chromosomes and spindles at MII stage after



**Fig. 2.** Detection of telomeres in wild-type (WT) and  $G_4$   $TR^{-}$  (TRKO) mouse oocytes. Telomere loss (arrows) was found at the ends of some chromosomes in  $TR^{-}$  mouse MI oocytes. Green, telomeres; blue, chromosomes.

meiotic maturation with those of wild-type mice. Twenty-nine (88%) of 33 GV oocytes from  $G_1$   $TR^{-}$  mice were matured to MII stage, with extrusion of a polar body (Figure 3A, arrowheads). Further, chromosomes aligned properly at the metaphase plates of intact spindles in these MII oocytes (Figure 3A, arrows), with



**Fig. 3.** Chromosomes, spindles and telomeres at metaphase of wild-type (WT) and  $G_1$   $TR^{-}$  (TRKO) mouse oocytes. **(A)** Chromosome alignments (arrows) at the metaphase plates of intact spindles from both WT and TRKO mouse MII oocytes. Green, spindles; red, chromosomes. Arrowheads indicate the first polar body. Scale bar, 10  $\mu$ m. **(B)** Telomere fluorescence signals at the chromosome ends of TRKO mouse oocytes. Green, telomeres; blue, chromosomes.

no lagging chromosomes along the spindles. As a control, in 27 oocytes analyzed from wild-type mice, 24 (89%) reached MII stage, and, except just one oocyte with one lagging chromosome, all the other 23 showed chromosome alignments at the metaphase plates of MII spindles, which appeared to be intact (Figure 3A). The number of chromosome ends lacking obvious telomere fluorescence signals did not differ between wild-type and  $G_1$   $TR^{-}$  mouse oocytes. Only 0.6% (five of 800) of the chromosome ends in five chromosome spreads of wild-type oocytes showed no apparent fluorescence signals, comparable to similar low percentages (0.4%, five of 1104) in lacking unambiguous telomere fluorescence signals at the chromosome ends of seven chromosome spreads analyzed from  $G_1$   $TR^{-}$  mouse oocytes (Figure 3B). Not unexpectedly, we did not find differences in meiotic maturation, chromosome alignments and spindle integrity between wild-type and  $G_1$   $TR^{-}$  oocytes, suggesting that telomerase deficiency itself does not cause defects in meiosis.

Ovary atrophy was observed in most  $G_4$   $TR^{-}$  mice that also show disrupted ovulation in spite of exogenous hormone stimulation, probably indicating defects in both germ cells and surrounding

somatic cells, which may have undergone extensive apoptosis with telomere shortening in the absence of telomerase. Apoptosis has been observed in late-generation  $TR^{-}$  male counterparts, testis, resulting in testicular atrophy and depletion of male germ cells (Lee *et al.*, 1998; Hemann *et al.*, 2001). To avoid complicated effects possibly originating from pre-antral follicles or follicles undergoing apoptosis and atresia, nude oocytes without surrounding cumulus cells were excluded from the present study.

Under natural conditions, telomere clustering at one pole of the nuclear envelope, an area known as spindle pole body in fission yeast and the centrosome in mammals, and chromosomal bouquet arrangement participate in the pairing and recombination of homologous chromosomes before the PI arrest stage (Scherthan *et al.*, 1996; de Lange, 1998; Scherthan *et al.*, 2000). Telomere loss and dysfunction could possibly have disrupted the pairing and recombination of homologous chromosomes during leptotene/zygotene stages of early PI. Telomere dysfunction may also directly prohibit organization and maintenance of functional meiotic spindles, particularly at the MII stage. This possibility may be partially supported by the observation of telomere loss in some chromosomes and free chromosome ends with no connection with microtubules. Further experiments are needed to examine whether misaligned chromosomes are those with telomere loss. It is possible that interactions between functional telomeres and microtubules facilitate spindle assembly and integrity, crucial for proper chromosome alignments.

We demonstrate that telomerase deficiency *per se* does not cause abnormal behavior of chromosomes and spindles at metaphases of meiosis. Instead, telomere loss and dysfunction lead to disruption of spindle and/or misalignment of metaphase chromosomes. The mechanisms underlying chromosome misalignments and disruption of spindles caused by telomere dysfunction are not yet known. Chromosome misalignment and spindle disruption could result from improper homologous pairing owing to telomere loss prior to PI. Alternatively, functional telomeres are directly involved in the spindle organization and mediate chromosome alignments at metaphase during meiotic division. Regardless of the mechanisms, it seems likely that maintaining proper alignment of chromosomes and spindle integrity requires involvement of functional telomeres to ensure equal separation of chromosomes during meiotic cell division. Our results may reveal a novel role of functional telomeres in the formation or maintenance of functional spindles and proper chromosome alignment at the metaphase plate.

## METHODS

**Animals, oocytes and *in vitro* culture.** Adult telomerase-null ( $TR^{-}$ ) mice and age-matched wild-type controls (Blasco *et al.*, 1997; Lee *et al.*, 1998) were primed with 5 IU pregnant mare's serum gonadotrophin (Calbiochem, La Jolla, CA). Forty-four hours later, GV oocytes enclosed in cumulus complexes were isolated from ovaries of 2–4-month-old wild-type,  $G_1$  and  $G_4$   $TR^{-}$  mice and cultured in MEM supplemented with 10% fetal bovine serum (Eppig and Telfer, 1993). All manipulations were carried out at 36–37°C on heated stages, chambers or in incubators. Unless specified, all reagents were obtained from Sigma (St Louis, MO). **Immunofluorescence staining and confocal microscopy.** Based on preliminary observations, oocytes were removed from cumulus cells by pipetting after brief incubation in 0.03%

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hyaluronidase and fixed at 9 h (mostly at MI stage) and 12–14 h (mostly early MII stage) of *in vitro* maturation. Oocytes were processed for indirect immunofluorescence with anti- $\beta$ -tubulin for staining microtubules as described previously (Liu *et al.*, 1998). Chromosomes were stained with propidium iodide. Images were captured with a Zeiss LSM 510 laser scanning confocal microscope or Nikon PCM 2000 confocal microscope.

**Analysis of telomeric function using quantitative fluorescence *in situ* hybridization (Q-FISH) with telomere probe.** Q-FISH has become the method of choice for examination of both telomere length and loss in single cells (Zijlmans *et al.*, 1997). Chromosome spreads were prepared by a hypotonic treatment of oocytes or spermatocytes with 1% sodium citrate for 20 min, followed by fixation in methanol:acetic acid (3:1), and air dried. FISH with FITC-labeled (CCCTAA)<sub>3</sub> PNA probe (Applied Biosystems, Framingham, MA) was performed according to the manufacturer's protocol. Chromosomes were counter-stained with 0.2  $\mu$ g/ml Hoechst 33342. Embryos were mounted onto a glass slide in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Telomeres were detected with a FITC filter using a Zeiss fluorescence microscope (AxioPlan 2 imaging), and images were captured by an AxioCam using AxioVision 3.0 software.

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