Tankyrase 2 Poly(ADP-Ribose) Polymerase Domain-Deleted Mice Exhibit Growth Defects but Have Normal Telomere Length and Capping

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Regulation of telomere length maintenance and capping are a critical cell functions in both normal and tumor cells. Tankyrase 2 (Tnks2) is a poly(ADP-ribose) polymerase (PARP) that has been shown to modify itself and TRF1, a telomere-binding protein. We show here by overexpression studies that tankyrase 2, like its closely related homolog tankyrase 1, can function as a positive regulator of telomere length in human cells, dependent on its catalytic PARP activity. To study the role of Tnks2 in vivo, we generated mice with the Tnks2 PARP domain deleted. These mice are viable and fertile but display a growth retardation phenotype. Telomere analysis by quantitative fluorescence in situ hybridization (FISH), flow-FISH, and restriction fragment analysis showed no change in telomere length or telomere capping in these mice. To determine the requirement for Tnks2 in long-term maintenance of telomeres, we generated embryonic stem cells with the Tnks2 PARP domain deleted and observed new change, even upon prolonged growth, in telomere length or telomere capping. Together, these results suggest that Tnks2 has a role in normal growth and development but is not essential for telomere length maintenance or telomere capping in mice.

Regulation of telomere length and protection of chromosome ends are two critical telomere functions that are essential in preventing premature senescence and in maintaining genome stability (reviewed in references 22 and 41). Mammalian telomeres consist of TTAGGG repeats that are bound by telomeric DNA repeat binding proteins and their associated factors, which together regulate telomere length maintenance and chromosome end protection (reviewed in references 17 and 56). TRF1, a double-stranded telomere repeat binding protein (12), functions as a negative regulator of telomere length by acting in cis to control access of telomerase (2, 58), a reverse transcriptase that uses an RNA template to add telomeric repeats (23, 24). Tankyrase 1 (Tnks1), which was identified in a two-hybrid screen with TRF1 (55), acts as a positive regulator of telomere length (54). Tankyrase 1 is comprised of several domains: the amino-terminal HPS domain, which consists of homopolymeric tracts of histidine, proline, and serine repeats; the large ANK domain, which is made up of 24 ankyrin repeats and comprises five functional subdomains (19, 50, 51); the sterile alpha module (SAM) domain, which is involved in tankyrase multimerization (18, 19, 50); and a poly(ADP-ribose) polymerase (PARP) domain (PD). The PARP domain of tankyrase 1 places this protein in the superfamily of PARP proteins.

PARPs utilize NAD+ as a substrate to synthesize long linear or branched polymers of ADP-ribose on protein acceptors (reviewed in references 1 and 52). Tankyrase 1 poly(ADP-riboseyl)ates TRF1 in vitro, inhibiting its ability to bind to telomeric DNA (55). Upon overexpression of tankyrase 1 in the nucleus, TRF1 is removed from telomeres (13, 54). The DNA-unbound form of TRF1 is ubiquitinated and degraded by the proteasome (10). Long-term overexpression of tankyrase 1 in human cells results in loss of TRF1 and telomere elongation, dependent on the catalytic PARP activity of tankyrase 1 (13, 54).

Another function of tankyrase 1 was revealed by knockdown of tankyrase 1 expression. Tankyrase 1 short interfering RNA (siRNA) treatment in human cells resulted in mitotic arrest with aberrant chromosome configurations and abnormal spindle structures (20). Sister chromatids were able to separate at centromeres and arms but were unable to separate at their telomeres. Use of siRNA-resistant wild-type (WT), but not PARP-dead, tankyrase 1 rescued this phenotype, indicating a requirement for tankyrase 1 PARP activity in sister telomere resolution and mitotic progression (20). More recent studies characterized spindle defects in tankyrase 1 siRNA cells and found defects in bipolar spindle formation and supernumerary spindles (8).

In addition to its telomeric localization (via TRF1 binding), tankyrase 1 localizes to other subcellular sites, including mitotic centromeres (53) and the Golgi apparatus (11), and has multiple binding partners. Tankyrase 1 has been shown to interact with IRAP (insulin-responsive aminopeptidase) in GLUT4 vesicles in the Golgi apparatus and further to be phosphorylated by mitogen-activated protein kinase upon insulin stimulation, suggesting a role for tankyrase 1 in mitogen-activated protein kinase-dependent regulation of GLUT4 vesicles (11). Tankyrase 1 has also been found to interact with and inhibit the Mcl-1 (myeloid cell leukemia 1) proteins, which function to regulate apoptosis (3). In addition, interactions of tankyrase 1 with FBP-17 (formin-binding protein 17) (21) and with TAB182 (tankyrase-binding protein of 182 kDa), a heterochromatin- and cortical actin-staining protein (51), have been observed. Finally, tankyrase 1 has been found to bind to
and colocalize with NuMA (nuclear and mitotic apparatus protein) at mitotic centrosomes (49, 53). Recent studies indicate that NuMA is a major acceptor of poly(ADP-ribosyl)ation by tankyrase 1 in mitosis (8, 9).

Tankyrase 2 (Tnks2; a closely related homolog) was identified by several groups through two-hybrid screens with IRAP (11); Grb14, an SH2 domain-containing adaptor protein that binds to the insulin receptor (IR) (40); and TRF1 (29), as well as by serological screens using a fetal brain cDNA library with meningioma patient sera (36). Studies indicate that there is twofold more tankyrase 1 than tankyrase 2 in DT40 chicken cells (19). The domains that comprise tankyrase 2 are highly homologous to those of tankyrase 1, with the main exception being that tankyrase 2 lacks the HPS domain. The tankyrase 2 ANK domain shares 83% identity with that of tankyrase 1, and as the ANK domain is responsible for protein-protein interactions, tankyrase 2 interacts with many of the same proteins as tankyrase 1, including TRF1 (13, 29), TAB182 (51), NuMA (49), and IRAP (11, 50). The tankyrase 2 SAM domain shares 74% identity with that of tankyrase 1, allowing both self-oligomerization and oligomerization with tankyrase 1 (18, 19, 50). The PARP domain is the most conserved, with 94% identity. Tankyrase 2 has been shown to have PARP activity similar to that of tankyrase 1 (13, 50) and can poly(ADP-ribosyl)ate itself and TRF1, releasing TRF1 from telomeres (13).

To further elucidate the role of tankyrase 2 at telomeres, we generated stable human cell lines overexpressing tankyrase 2 in the nucleus. We show that tankyrase 2 induces telomere elongation, dependent on its catalytic PARP activity. Our results confirm that, as expected from the high degree of homology between the two proteins, tankyrase 2 (like tankyrase 1) can influence telomere length in human cells. Next, to study the function of tankyrase 2 on telomere length in vivo, we generated a tankyrase 2 PARP domain-deleted mouse. Characterization of this mouse revealed a small mouse phenotype, and telomere analysis showed no change in telomere length and no defect in telomere capping. Thus, in mice, a PARP-active Tnks2 is not required for telomere maintenance but is necessary for normal growth.

**MATERIALS AND METHODS**

**Plasmids.** Full-length tankyrase 2 (amino acids 2 to 1166) with an amino-terminal Myc tag and nuclear localization sequence (MN-TNKS2.WT) was cloned as described previously (13). MN-TNKS2.HE/A was generated by replacing the conserved active site histidine and glutamic acid residues at positions 1031 and 1138, respectively, with alanine residues by site-directed mutagenesis of the conserved active site histidine and glutamic acid residues at positions 155 and 213 (55) proteins was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transferred to a nitrocellulose membrane. Blots were incubated with the following primary antibodies: rabbit anti-Myc (0.7 μg/ml; Santa Cruz Biotechnology), rabbit anti-TRF1 415 (0.5 μg/ml) (13), rabbit anti-tankyrase 2 608 (0.7 μg/ml), rabbit anti-b-actin (0.2 μg/ml; Santa Cruz Biotechnology), and rabbit anti-tankyrase 1 465 (1.8 μg/ml) (55). Immunoprecipitation of cell or tissue extracts was performed as described previously (13) using 1.8 μg/ml rabbit anti-tankyrase 1 465.

**Construction of mTnks2 targeting vector.** The targeting vector was designed to replace exons 25, 26, and 27 (PARP domain of Tnks2) with a neomycin (neo) resistance cassette. (Note that the mTnks2 exon nomenclature that is used in this paper follows that described in reference 29. The predicted exon structure for mTnks2 in the GenBank and Mouse Genome Informatics databases includes one additional exon upstream of exon 1.) The cloned phosphoglycerate kinase-neo cassette from the pPNeo5neo vector (kindly provided by Alexandra Joyner, Skirball Institute) was cloned into pSP72 (from Promega, with a NotI site added to the P6 site). The 1.5-kb short arm was generated by PCR from mouse 129/SvEv genomic DNA using primers 5'-GTGGGAAAGATACACCCGGAG-3' and 5'-GTGGACATCTGCACTGAG-3'. The short arm began within exon 24 and extended to 90 bp upstream of exon 25 and was inserted 5' of the neo cassette. The long arm, which began at the XbaI site downstream of exon 27 and extended 7.5 kb, was generated from a large phage clone isolated from a mouse 129/SvEv genomic library and was inserted 3' of the neo cassette.

**Targeted disruption of the mTnks2 gene in ES cells.** Ten micrograms of the targeting vector was linearized with NotI and electroporated into 129/SvEv ES cells. After selection in G418 (200 μg/ml), surviving colonies were expanded, and PCR analysis was performed to identify clones that had undergone homologous recombination. To detect the wild-type allele, the following primer pair was used: primer 1, 5'-GTCGGGAAAAGATACACCCGGAG-3'; and primer 2, 5'-AAA TCCGAGTCCAACACATGCAC-3'. For the mutant allele, the following primer pair was used: primer 3, 5'-GACACTTCGGCAAGTTGGTT-3'; and primer 4, 5'-TGGAGGCGGAAGGACATTTGATAG-3'. The PCR conditions were 94°C for 5 min; followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 7 min.

**Construction of Tnks2G297Asn mice.** Tnks2G297Asn null (−/−) mice were crossed to C57BL/6 mice to remove the Tk-Cre transgene (kindly provided by Alexandra Joyner, Skirball Institute [5]) to remove the floxed neo cassette. Tnks2G297Asn+/− TK-Cre mice were backcrossed to C57BL/6 mice to remove the TK-Cre transgene. These Tnks2G297Asn+/− mice were backcrossed again to C57BL/6 mice before breeding Tnks2G297Asn+/− mice to generate Tnks2G297Asn−/− mice. Genotyping was performed using PCR primers 1 and 2 (see above) for the wild-type allele and primers 1 and 5 for the mutant, neo-deleted allele, using the same PCR conditions as used above (PCR primer 5, 5'-GAGGGTTCAAGAGGCTGCGTATG-3'). Southern blotting was performed using the probe described above.

**Northern blotting.** Total RNA was prepared from MEFs using the RNaseasy Mini kit (QIAGEN). Blots were probed with DNA probes corresponding to amino acids 811 to 896 for human tankyrase 2 and 336 to 1163 for human tankyrase 2 amino acids 811 to 896.
The mean telomere fluorescence of cells gated at G0/G1 was analyzed with CellQuest 2 h before acquisition on the FACScan (Becton Dickinson) flow cytometer. /H9262 buffered saline, 10 U/ml RNase A, 0.1% BSA, 0.1% image on the chromosome image using Photoshop software.

Quantitative fluorescence in situ hybridization (Q-FISH). Spleens from wild-type and Tnks2+/−/− littermate mice, or wild-type and Tnks2+/ΔΔneo−/− littermate mice, were used to prepare single-cell suspensions by mincing samples through a 70-μm nylon cell strainer (BD Biosciences). Splenocytes were cultured in DMEM-10% FBS and activated with 1 μg/ml concanavalin A (Sigma) and 1 μg/ml phytohemagglutinin (Sigma) for 3 h before cells were arrested in metaphase with a 6-h treatment with 0.1 μg/ml Colcemid (Gibco). Cells were collected, and metaphase spreads were prepared from splenocytes as described previously (20) and from ES cells as described previously (61). Images were acquired on a Zeiss Axioplan 2 microscope with a Photometrix SenSyn camera. Photographs were processed using OpenLab software. TFL-TELO software (kindly provided by P. Landsorp, Terry Fox Laboratory, Vancouver, Canada) was used to quantify the telomere signal from 10 metaphases for each sample.

Flow-FISH. Spleen and thymus from wild-type and Tnks2+/−/− littermate mice or wild-type and Tnks2+/ΔΔneo−/− littermate mice were used to prepare single-cell suspensions of splenocytes and thymocytes by mincing samples through a 70-μm nylon cell strainer. Erythrocytes were removed from the cell suspension by lysis with ACK lysing buffer, and samples were frozen at −80°C until processed for flow-FISH. Frozen samples were thawed and washed two times in DMEM. Viable cells were counted and resuspended in phosphate-buffered saline–0.1% /H9262 suspensions of splenocytes and thymocytes by mincing samples through a 70-μm nylon cell strainer. Erythrocytes were removed from the cell suspension by lysis with ACK lysing buffer, and samples were frozen at −80°C until processed for flow-FISH. Frozen samples were thawed and washed two times in DMEM. Viable cells were counted and resuspended in phosphate-buffered saline–0.1% /H9262.

Viable cells were counted and resuspended in phosphate-buffered saline–0.1% /H11002 of the indicated genotypes, cells were embedded in agarose plugs and digested with HinfI and RsaI, DNA fragments were separated by pulsed-field gel electrophoresis (CHEF DR-II apparatus, Bio-Rad), and telomere restriction fragments (TRFs) were scored with TELO software (Fox Chase Cancer Center). For primary or immortalized MEFs with Tnks2PD+/− or Tnks2PD+/ΔΔneo−/−, DNA was denatured at 86°C for 10 min, and the samples were hybridized with H9262 conjugated (TTAGGG)3 peptide nucleic acid (PNA) probe (Applied Biosystems).

RESULTS

Human tankyrase 2 is a positive regulator of telomere length. Previous studies indicated that tankyrase 2 shared a number of properties with tankyrase 1. Both were found to bind TRF1 and poly(ADP-riboseyl)ate TRF1 in vitro (13, 29, 55). Overexpression studies of tankyrase 1 showed that when it was expressed in the nucleus (via an amino-terminal nuclear localization signal) it removed TRF1 from telomeres (whereupon TRF1 was degraded by the proteasome) and, over the long term, induced telomerase-dependent telomere elongation (10, 13, 54). The activities of tankyrase 1 were dependent on its catalytic PARP activity, since a catalytically dead allele (HE/A) (generated in the same way as Tnks2+/ΔΔneo−/− littermate mice) had no effect on telomere elongation (Fig. 1B and C) and was similar to a vector control (data not shown). Together these studies indicate that tankyrase 1 (like tankyrase 2) can act as a positive regulator of telomere length in human cells dependent on its catalytic PARP activity.

To determine if tankyrase 2 is required to maintain telomere length in vivo, we sought to delete the tankyrase 2 gene (Tnks2) in mouse. In order to monitor tankyrase 2 protein expression, we raised tankyrase 2-specific antibodies against an E. coli-expressed fusion protein containing human tankyrase 2 amino acids 811 to 896 (Fig. 1D). The specificity of the antibody was determined by immunoblotting against baculovirus-expressed human tankyrase 1 or tankyrase 2. As shown in Fig. 1E, anti-tankyrase 2 608 specifically detected tankyrase 2 (lane 4) but not tankyrase 1 (lane 3). By contrast, a previously generated antibody to tankyrase 1 (465) (55) detected both tankyrase 1 (lane 5) and tankyrase 2 (lane 6).

Generation of Tnks2PD−/− and Tnks2+/ΔΔneo−/− mice. Tnks2 is located on mouse chromosome 19qC2. The gene is comprised of 27 exons. (See note in Materials and Methods.) The last four exons (24 through 27) encode the carboxy-terminal catalytic PARP domain of tankyrase 2 (Fig. 2A). We sought to disrupt the Tnks2 gene and create a null allele by deleting exons 25 through 27 (encoding carboxy-terminal amino acids 1032 through 1166 of mTnks2) and replacing them with the neo resistance gene (neo). However, as described below, the targeted disruption did not result in a null allele but rather in expression of truncated forms of Tnks2. The truncated proteins lack the catalytic PARP domain; hence, the targeted allele will be referred to as Tnks2PD−/− (PARP domain deleted) (Fig. 2A).

Mouse ES cells (129/SvEv) were electroporated with the linearized targeting construct and selected for G418 resistance. Clones were screened using the PCR strategy described below. Chimeric mice (generated from injection of a positive ES cell clone into a blastocyst) were mated to C57BL/6 for germ line transmission to generate Tnks2PD−/+ mice.

Mating of Tnks2PD−/+ mice generated Tnks2PD−/− mice, as shown by Southern blotting and PCR analysis of tail DNA. For Southern blot analysis, to monitor the targeting of the Tnks2 locus, we made use of a ScaI site in the neo gene, which generates a 5.1-kb ScaI fragment that can be distinguished from the 4.3-kb ScaI fragment in the wild-type allele (Fig. 2A and B). The genotype of the mice was further confirmed using PCR analysis (Fig. 2A and C). For the wild-type allele, PCR was performed with a 5’ primer in exon 24 and a 3’ primer in exon 25, which resulted in a 1.7-kb fragment specific to the wild-type allele. For the targeted allele PCR was performed with a 5’ primer upstream of exon 24 (not found in the targeting vector) and a 3’ primer in the neo gene, which resulted in a 2.0-kb fragment specific to the targeted allele.

Consistent with targeting of the Tnks2 locus, Northern blot analysis of primary MEFs showed no detectable Tnks2 RNA directed mutagenesis. As shown in Fig. 1A, overexpression of wild-type (lane 2) but not PARP-dead (lane 3) tankyrase 2 resulted in reduced levels of TRF1. Telomere length analysis showed that MN-tankyrase 2.2WT induced telomere elongation (Fig. 1B). Telomerizes showed progressive elongation at a rate of approximately 38 bp per population doubling (pd) (Fig. 1C), similar to tankyrase 1 (13, 54). By contrast, overexpression of MN-tankyrase 2.HE/A had no effect on telomere elongation (Fig. 1B and C) and was similar to a vector control (data not shown). Together these studies indicate that tankyrase 2 (like tankyrase 1) can act as a positive regulator of telomere length in human cells dependent on its catalytic PARP activity.
Moreover, we did not detect any major new transcripts in Tnks2PD/H9004/H11001/H11002 or Tnks2PD/H9004/H11002/H11002 cells. As expected, immunoblot analysis of immortalized MEFs indicated that wild-type (full-length) Tnks2 was not expressed in Tnks2PD/H9004/H11002/H11002 cells (Fig. 2E). However, alternative Tnks2 proteins (which were not found in wild-type cells) were expressed in Tnks2PD/H9004/H11001/H11002 and Tnks2PD/H9004/H11002/H11002 cells, indicating that deletion of exons 25 through 27 did not result in complete ablation of Tnks2 protein expression. Our failure to detect alternative transcripts by Northern blotting could be due to their lower abundance or stability.

We observed two classes of alternative Tnks2 proteins in Tnks2PD+/− or Tnks2PD−/− cells. As expected, immunoblot analysis of immortalized MEFs indicated that wild-type (full-length) Tnks2 was not expressed in Tnks2PD−/− cells (Fig. 2E). However, alternative Tnks2 proteins (which were not found in wild-type cells) were expressed in Tnks2PD+/− and Tnks2PD−/− cells, indicating that deletion of exons 25 through 27 did not result in complete ablation of Tnks2 protein expression. Our failure to detect alternative transcripts by Northern blotting could be due to their lower abundance or stability.

The faster-migrating proteins likely corresponded to truncated Tnks2 proteins. The targeted Tnks2 allele lacks the carboxy-terminal 134 amino acids. Thus, a truncated protein would be predicted to migrate with an apparent molecular mass of 114 kDa (compared to 128 kDa for wild-type Tnks2), consistent with the protein analysis in Fig. 2E. The truncated Tnks2 proteins likely contain the ANK and SAM domains, which are important for scaffolding functions and protein-protein interactions. However, the alternative Tnks2 proteins lack the catalytic PARP domain and are therefore PARP dead.

We reasoned that the slower-migrating class of proteins may have resulted from splicing of a truncated Tnks2 to downstream sequences, in particular neo. Other groups have re-
ported generating neo fusion proteins when attempting targeted gene disruption with the neo cassette and have noted the presence of cryptic splice sites within the cassette (7, 28). As the neo gene in the targeted allele was flanked by loxP sites (Fig. 2A), we addressed this possibility by deleting the neo gene from the targeted allele using Cre recombinase. Thus, we crossed Tnks2PDΔ/neoΔ mice to C57BL/6 mice carrying a TK-Cre transgene. The Tnks2PDΔneoΔ+/− TK-Cre mice were backcrossed to C57BL/6 mice to remove TK-Cre. These Tnks2PDΔneoΔ+/− mice were backcrossed again to C57BL/6 mice before breeding heterozygous mice to generate Tnks2PDΔneoΔ−/− mice. To monitor excision of the neo allele, we took advantage of replacement of the Scal site in the neo gene with a downstream Scal site which resulted in a 5.9-kb Scal fragment, distinguishing the PDΔneoΔ allele from the
wild-type allele (Fig. 2A and F). The genotype of the mice was further confirmed using PCR analysis (Fig. 2A and G). For the wild-type allele, PCR was performed (as described above) with a 5′ primer in exon 24 and a 3′ primer in exon 25, which resulted in a 1.7-kb fragment specific to the wild-type allele. For the neo-deleted allele PCR was performed with a 5′ primer upstream of exon 24 and a 3′ primer just 3′ to the remaining loxP site, which resulted in a 1.72-kb fragment specific to the neo-deleted allele. As shown by immunoblot analysis of brain tissue, the high-molecular-weight neo product was no longer expressed in the Tnks2PDΔ/Δ−/− mice; only the low-molecular-weight, truncated neo product was detected (Fig. 2H).

Small size in Tnks2PDΔ/− and Tnks2PDΔneoΔ−/− mice. As described above, mating of Tnks2PDΔ+/+ mice generated Tnks2PDΔ/− mice at the expected Mendelian ratio (37 Tnks2PDΔ/− mice, 69 Tnks2PDΔ/−/− mice, and 35 Tnks2PDΔ+/+ mice). Tnks2PDΔ/− mice (both male and female) were viable and fertile. However, a small but significant fraction died within 20 days of birth (7/37 [19%] Tnks2PDΔ/− mice, 1/69 [1%] Tnks2PDΔ/−/− mice, and 2/35 [6%] Tnks2PDΔ+/+ mice). The most striking feature of the Tnks2PDΔ/− mice was their smaller body size (Fig. 3A). We monitored the weight of the mice from birth to 8 weeks. As shown in Fig. 3B, Tnks2PDΔ/− pups showed a weight similar to that of their wild-type and Tnks2PDΔ/− littermates 2 and 4 days after birth. However, by 6 days Tnks2PDΔ/− pups weighed less than their littermates. The difference was most dramatic at 20 days (Tnks2PDΔ/− mice were 40% smaller than Tnks2PDΔ+/+ mice) but decreased over time (at 56 days Tnks2PDΔ/− mice were 20% smaller than Tnks2PDΔ/− and Tnks2PDΔ+/+ mice). The weights of heart, brain, lungs, testis, kidneys, and liver were proportionately reduced in size, but thymus and spleen were variable (Fig. 3C). We also observed a few cases of severely small Tnks2PDΔ/− mice that were 50 to 60% smaller than Tnks2PDΔ+/+ and Tnks2PDΔ+/+ mice; however, these mice tended to die before weaning and were not included in the growth curve or organ size analysis.

Tnks2PDΔneoΔ−/− mice exhibited a similar small mouse phenotype (Fig. 3D and E), indicating that the phenotype was consistent in both the Tnks2PDΔ/− and Tnks2PDΔneoΔ−/− mice. However, compared to the Tnks2PDΔ mice, we noticed an increase in death before 20 days of age in both Tnks2PDΔneoΔ−/− mice and Tnks2PDΔneoΔ−/− mice (6/35 [18%] Tnks2PDΔneoΔ−/− mice and 11/20 [55%] Tnks2PDΔneoΔ−/− mice) and an approximately twofold increase (data not shown) in the number of severely
TABLE 1. Measurement by flow-FISH of telomere length in splenocytes and thymocytes obtained from Tnks2PDA (−/−) and Tnks2PDAneoΔ (−/−) mice and their wild-type (+/+ or heterozygous (+/−) littermates

| Mouse type       | Litter | Mouse no. | Genotype     | Telomere lengthb |
|------------------|--------|-----------|--------------|------------------|
| Tnks2PDA         | 1      | M395      | +/+          | Spleen 1.44 0.93 |
|                  |        | M396      | −/−          | Thymus 1.42 1.04 |
|                  |        | F97        | +/+          | Spleen 1.63 0.80 |
|                  |        | F399       | +/+          | Thymus 1.67 0.70 |
|                  |        | F401       | −/−          | Spleen 0.96 0.97 |
|                  |        | F402       | +/+          | Thymus 1.53 0.59 |
|                  | 2      | M443       | +/+          | Spleen 0.53 0.98 |
|                  |        | M446       | −/−          | Thymus 0.62 1.37 |
|                  |        | F448       | −/−          | Spleen 0.96 1.74 |
|                  |        | F450       | +/+          | Thymus 0.99 0.84 |
|                  | 3      | F467       | +/+          | Spleen 0.56 0.66 |
|                  |        | F468       | −/−          | Thymus 0.83 1.04 |
| Tnks2PDAneoΔ     | 1      | F502       | +/+          | Spleen 0.77 0.88 |
|                  |        | F501       | +/+          | Thymus 1.01 1.06 |
|                  |        | F559       | +/+          | Spleen 1.60 1.26 |
|                  |        | F560       | −/−          | Thymus 1.08 1.05 |
|                  | 2      | M628       | +/+          | Spleen 0.95 0.87 |
|                  |        | M629       | +/+          | Thymus 0.82 0.98 |
|                  | 1      | F600       | +/+          | Spleen 1.28 0.95 |
|                  |        | F601       | −/−          | Thymus 0.92 0.82 |
|                  |        | F602       | +/+          | Spleen 0.98 0.83 |
|                  |        | F604       | +/+          | Thymus 0.79 1.15 |

a M, male; F, female.
b Telomere length is expressed in arbitrary fluorescent units. For Tnks2PDA mice, average telomere fluorescence was 0.88 (+/+ versus 1.13 (−/−) in splenocytes (P = 0.19) and 0.97 (+/+ versus 1.05 (−/−) in thymocytes (P = 0.29). For Tnks2PDAneoΔ mice, average telomere fluorescence was 1.07 (+/+ versus 1.05 (−/−) versus 0.94 (−/−) in splenocytes (P = 0.42) and 1.03 (+/+ versus 0.97 (−/−) in thymocytes (P = 0.57).

As shown in Table 1 there was no significant difference between Tnks2PDAneoΔ (+/+ or −/−) and Tnks2PDAneoΔ mice. In agreement, Q-FISH analysis (Fig. 4D) indicated that telomere length distribution was similar in Tnks2PDAneoΔ (+/+ or −/−) mice.

Normal telomere capping in Tnks2PDA and Tnks2PDAneoΔ mice. To study the effect of the Tnks2 PARP domain deletion on telomere function, we analyzed 100 metaphase cells prepared from splenocytes from Tnks2PDA (+/+ and Tnks2PDAneoΔ mice by Q-FISH for chromosomal aberrations. We did not detect any change in signal free ends, end-to-end fusions, or chromosome breakage (Table 2).

Telomere length is maintained during prolonged culture of Tnks2PDAneoΔ ES cells. Previous studies have shown that, in ES cells deficient in telomerase, telomeres undergo shortening and show an increase in end-to-end fusions (37, 38, 43, 44). This phenotype is more apparent as ES cells are continually passaged. To determine if a deficiency in Tnks2 might influence telomere function during long-term growth, we generated Tnks2PDAneoΔ ES cell clones from a G418-resistant Tnks2PDAneoΔ ES cell clone by culture at increased G418 concentration. Clones were screened using the same PCR strategy described for Fig. 2C, and two Tnks2PDAneoΔ ES cell clones (A3 and C10) were isolated (Fig. 5A).

Immunoblot analysis indicated that (like Tnks2PDAneoΔ mice) Tnks2PDAneoΔ ES cells were deficient in wild-type Tnks2 protein but did express truncated (PARP-deleted) Tnks2 alone and fused to neo (Fig. 5B). We used Q-FISH to examine telomeres in Tnks2PDAneoΔ ES cells. As shown in Fig. 5C and D we did not detect telomere shortening in Tnks2PDAneoΔ ES cell clone A3 or C10 upon prolonged cell culture (from pd 42 to pd 212). In fact, the Tnks2PDAneoΔ mice showed a slight increase in telomere length during long-term growth, as has been reported previously for wild-type ES cells (37). Note that Tnks2PDAneoΔ ES cell clones had longer telomeres than Tnks2PDAneoΔ ES cells even at pd 42. This is likely due to the additional pd’s that the Tnks2PDAneoΔ ES cell clones underwent during G418 selection. Regardless, all three lines showed a similar increase in telomere length over 170 pd’s (WT, 28%; A3, 29%; C10, 26%), indicating that the Tnks2 PARP domain deletion had no effect on telomere length maintenance during prolonged cell culture.

To study the effect of the Tnks2 PARP domain deletion on telomere capping during prolonged cell culture, we analyzed 50 metaphase cells prepared from Tnks2PDAneoΔ and Tnks2PDAneoΔ ES cell cultures at 42 versus 212 pd’s for chromosomal aberrations. We did not detect any change in signal free ends, end-to-end fusions, or chromosome breakage (Table 3).

**DISCUSSION**

Tankyrase 2 and telomere function in mice. We have shown here that tankyrase 2 (like its closely related homolog tankyrase 1) can act as a positive regulator of telomere length when over-expressed in human cells (Fig. 1A to C). Telomere elongation by tankyrase 2 was found to be dependent on its catalytic PARP activity.
activity. To determine if tankyrase 2 was required for telomere maintenance in vivo, we analyzed telomere function in mice containing a PARP domain-deleted Tnks2. Telomere length analysis using three different methods (Q-FISH, flow-FISH, and telomere restriction fragment analysis) revealed no change in telomere length in Tnks2 PARP domain-deleted mice (Fig. 4; Table 1). Moreover, Tnks2 PARP domain-deleted ES cells, which were grown for 212 pd’s, showed no telomere shortening (Fig. 5). Together these data indicate that a PARP-active Tnks2 is not required for telomere length maintenance in vivo in mice and during long-term growth of mouse cells.

How can we reconcile the lack of requirement for PARP-active tankyrase 2 in telomere length maintenance in mice with
our demonstration that a PARP-active tankyrase 2 induces telomere elongation in human cells? Our studies, showing that tankyrase 2 (or tankyrase 1) can induce telomere elongation in human cells, are based on overexpression analysis. By contrast, our studies with mice utilize deletion analysis. Thus, it is possible that, in the absence of a catalytically active Tnks2, a redundant role for Tnks1 in telomere function is revealed, i.e., Tnks1 function is sufficient to maintain telomere length in the absence of Tnks2. Ultimately, a double knockout mouse, deficient in both Tnks1 and Tnks2, will be needed to address this question.

Other PARPs and telomeres. Other PARPs besides tankyrases 1 and 2 have been suggested to play a role at telomeres.

Table 3. Analysis of chromosomal abnormalities in Tnks2<sup>PΔ</sup> (−/−) and wild-type (+/+ ) ES cells

| Genotype   | Cell line<sup>a</sup> | pd | No. of metaphases | Signal free ends/total chromosomes<sup>b</sup> | End-to-end fusions/total chromosomes | Chromosome breakages/total chromosomes |
|------------|------------------------|----|-------------------|--------------------------------------------|--------------------------------------|---------------------------------------|
| Tnks2<sup>PΔ</sup> (+/+ ) | WT                      | 42 | 50                | 0/1,988                                    | 0/1,988                              | 0/1,988                               |
| Tnks2<sup>PΔ</sup> (+/+ ) | WT                      | 212| 50                 | 0/1,992                                    | 2/1,992                              | 2/1,992                               |
| Tnks2<sup>PΔ</sup> (−/−) | A3                      | 42 | 50                 | 0/2,056                                    | 0/2,056                              | 0/2,056                               |
| Tnks2<sup>PΔ</sup> (−/−) | A3                      | 212| 50                 | 0/2,083                                    | 1/2,083                              | 1/2,083                               |
| Tnks2<sup>PΔ</sup> (−/−) | C10                     | 42 | 50                 | 0/2,043                                    | 1/2,043                              | 1/2,043                               |
| Tnks2<sup>PΔ</sup> (−/−) | C10                     | 212| 50                 | 0/1,985                                    | 3/1,985                              | 0/1,985                               |

<sup>a</sup> A3 and C10 are independently derived Tnks2<sup>PΔ</sup> (−/−) ES cells.

<sup>b</sup> For detection of signal free ends, the telomere images were overexposed to intensify weak telomeric fluorescent signals.
PARP-1, the first identified member of the PARP family, is a DNA damage sensor and has also been implicated in many other cellular processes (reviewed in references 1 and 35). One report of PARP-1−/− mice showed a 30% decrease in telomere length and end-to-end fusions in 25% of cells (15), whereas another group using a different PARP-1−/− mouse found normal telomere length and chromosomal end capping (48). PARP-2, a homolog of PARP-1, has also been reported to play a role at telomeres. PARP-2 was shown to bind and poly(ADP-ribose)late TRF2 (16), a telomeric DNA binding protein that has a protective function (59). PARP-2−/− cells showed normal telomere length, but an increase in chromosome/chromatid breaks and signal free ends was observed (16). Another PARP with a potential telomeric function is vault PARP (VPARP) (34). VPARP is a component of the vault complex, a large (13-MDa), highly conserved, and ubiquitously expressed ribonucleoprotein complex (reviewed in reference 60). Other components of the vault complex include major vault protein, vault RNA, and telomerase-associated protein 1 (TEPI), which binds telomerase RNA (25). Studies of VPARP−/− mice and TEPI−/− VPARP−/− mice, however, showed normal telomere length and structure (39). While it is possible that PARP-1, PARP-2, and VPARP could have functions redundant with tankyrases 1 and 2 in maintaining normal telomere length and capping of telomeres, we think it unlikely, since the only homology between these three PARPs and tankyrases 1 and 2 is in the PARP catalytic domain.

Tankyrase 2 and postnatal growth in mice. Tnks2 PARP domain-deleted mice are generated at the expected Mendelian ratios, suggesting that Tnks2 PARP activity is not required for embryonic development. Indeed, Tnks2 PARP domain-deleted neonates are indistinguishable from WT littersmates from birth until postnatal day 6. However, after day 6 growth retardation becomes apparent (Fig. 3B). The Tnks2 PARP domain-deleted mice were, on average, 20% smaller than WT or heterozygous littermates (Fig. 3A, B, and D). While we do not know the mechanism for this growth defect, we speculate (based on known tankyrase 2 binding partners) that tankyrase 2 could play a role in insulin-mediated effects on postnatal growth (see below).

Thus, tankyrase 2 has been shown to bind to Grb14 (40), a member of the Grb7/10/14 family of adaptors (reviewed in reference 27). Grb14 binds activated IR (30) and inhibits the phosphorylation of IR substrates (6). Overexpression of Grb14 has been shown to inhibit the activation of downstream insulin signaling cascades (6, 26, 30). PARP-2, one of the tankyrases (13-MDa), highly conserved, and ubiquitously expressed ribonucleoprotein complex (reviewed in reference 60). Other components of the vault complex include major vault protein, vault RNA, and telomerase-associated protein 1 (TEPI), which binds telomerase RNA (25). Studies of VPARP−/− mice and TEPI−/− VPARP−/− mice, however, showed normal telomere length and structure (39). While it is possible that PARP-1, PARP-2, and VPARP could have functions redundant with tankyrases 1 and 2 in maintaining normal telomere length and capping of telomeres, we think it unlikely, since the only homology between these three PARPs and tankyrases 1 and 2 is in the PARP catalytic domain.

In addition to Grb14, IRAP is another protein in the insulin pathway that has been shown to bind to tankyrase 1 or 2 and to be an acceptor of poly(ADP-ribose)ylation (50). IRAP colocalizes with GLUT4, an insulin-responsive glucose transporter in adipocytes and muscle (46). After insulin stimulation, both IRAP and GLUT4 translocate from vesicles in the cytoplasm and trans-Golgi network to the membrane plasma (reviewed in reference 32). In IRAP−/− mice, absence of IRAP resulted in decreased GLUT4 expression (33). It has been suggested that tankyrases 1 and 2 may have a role in targeting or maintenance of GLUT4 vesicles (11, 50). Interestingly, GLUT4−/− mice (similar to our Tnks2 PARP-deleted mice) display growth retardation and are 20% smaller than controls (31). However, since IRAP−/− mice do not have decreased body weight (33), the role of the IRAP-tankyrase 2 interaction in the small mouse phenotype remains to be determined.

Our studies of Tnks2 PARP domain-deleted mice have shed some light on the different functions of Tnks2 in vivo. For telomere maintenance, Tnks1 and Tnks2 could share redundant functions, but for normal postnatal growth, Tnks1 is not sufficient, as our mice display growth retardation. Further studies of these mice and of mice deficient in both Tnks1 and Tnks2 will be valuable in distinguishing the functions of Tnks1 and Tnks2 in telomere length maintenance and growth.

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