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Telomeric TERB1-TRF1 interaction is crucial for male meiosis

Juanjuan Long^{#1,2,3}, Chenhui Huang^{#4,5}, Yanyan Chen^{1,2,3}, Ying Zhang^{1,2}, Shaohua Shi^{1,2}, Ligang Wu^{1,2}, Yie Liu⁶, Chengyu Liu⁷, Jian Wu^{4,5}, and Ming Lei^{4,5,8}

¹National Center for Protein Science Shanghai, State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

²Shanghai Research Center, Chinese Academy of Sciences, Shanghai, China

³University of Chinese Academy of Sciences, Shanghai, China

⁴Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

⁵Shanghai Institute of Precision Medicine, Shanghai, China

⁶Laboratory of Molecular Gerontology, National Institute on Aging/National Institute of Health, Baltimore, Maryland, USA

⁷Transgenic Core Facility, NHLBI, NIH, Bethesda, Maryland, USA

⁸Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, China

These authors contributed equally to this work.

Abstract

During meiotic prophase, the meiosis-specific telomere-binding protein TERB1 regulates chromosome movement required for homologous pairing and recombination by interacting with the telomeric shelterin subunit TRF1. Here, we report the crystal structure of the TRF1-binding motif of human TERB1 in complex with the TRFH domain of TRF1. Notably, specific disruption of the TERB1-TRF1 interaction by a point mutation in the mouse *Terb1* gene results in infertility only in males. We find that this mutation causes an arrest in the zygotene-early pachytene stage and mild telomere abnormalities of autosomes but unpaired X and Y chromosomes in pachytene, leading to massive spermatocyte apoptosis. We propose that the loss of telomere structure mediated by the TERB1-TRF1 interaction significantly affects homologous pairing of the telomere-adjacent pseudoautosomal region (PAR) of the X and Y chromosomes in mouse

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Correspondence should be addressed to M.L. (leim@shsmu.edu.cn) or J.W. (wujian@shsmu.edu.cn).

AUTHOR CONTRIBUTIONS

J.L., C.H., J.W., and M.L. designed the study. J.L. crystallized the TERB1_{TBM}-TRF1_{TRFH} complex. J.W. collected X-ray data and determined the crystal structure. J.L., C.H., Y.C., and S.S. carried out biochemical assays. J.L., Y.L., C.L., Y.Z., and L.W. participated in the generation of the knock-in mice. J.L., C.H., and Y.C. performed the experiments to characterize the meiotic phenotype. C.H., J.W., and L.M. drafted the manuscript. All authors reviewed and edited the manuscript.

COMPETING FINANCIAL INTERESTS

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spermatocytes. Our findings uncover a specific mechanism of telomeres that surmounts the unique challenges of mammalian X-Y pairing in meiosis.

Meiosis is a specialized cell division for gametogenesis that contributes to sexual reproduction by reducing the diploid chromosome number to the haploid¹. During meiotic prophase I, homologous chromosomes undergo homolog pairing, synapsis, and reciprocal recombination, which are crucial for the chromosome segregation during metaphase I (refs. 1-3). In most organisms studied to date, including mammals, repair of the programmed DNA double-stranded breaks (DSBs) introduced by SPO11 in leptotene initiates a genomewide search for homology⁴⁻⁷. This search drives the homolog pairing and alignment, leading to assembly of a proteinaceous structure called the synaptonemal complex along the length of the paired homologs^{8,9}. The DSBs can be repaired either as a crossover or as a noncrossover^{7,8}, the former resulting in chiasmata that are essential for the correct alignment and segregation of homologous chromosomes during metaphase I (refs. 10-12). Despite the importance of SPO11-mediated DSBs in mammalian meiotic synapsis, a significant level of homolog pairing at chromosomal ends was detected in mouse spermatocytes before programmed DSBs¹³. This is consistent with the observation that synapsis appears to initiate in subtelomeric regions in human spermatocytes¹⁴, suggesting a positive role of telomeres and subtelomeres in the initiation of meiotic homolog pairing.

In recent years, mounting evidence has indicated that progression of mammalian meiotic prophase I depends on the telomere-led rapid prophase movements of chromosomes along the nuclear envelope, which has been observed in diverse eukaryote species¹⁵⁻¹⁷. A prerequisite for this rapid chromosome movement is the attachment of telomeres to the nuclear envelope, where the transmembrane LINC (linker of nucleoskeleton and cytoskeleton) complex serves as a structural bridge to connect telomeres to the cytoskeleton and transduce forces generated in the cytoplasm to the end of the chromosomes^{17,18}. In addition to the LINC-complex protein SUN 1 (ref. 19), several meiosis-related molecules also play important roles in mediating telomere-nuclear envelope attachment in mice, including TERB1 and TERB2 (telomere-repeat-binding bouquet-formation proteins 1 and 2)²⁰⁻²², MAJIN (membrane-anchored junction protein)²², CDK2 (cyclin-dependent kinases 2)²³ and Speedy/RINGO A (rapid inducer of G2/M progression in oocytes)²⁴.

The meiosis-specific telomere regulator TERB1 is a molecular scaffold that simultaneously interacts with SUN1, TERB2, meiotic cohesin subunit SA3, and telomeric shelterin subunit TRF1, establishing telomere attachment to the inner nuclear membrane (INM) and driving the chromosome movement required for homologous pairing and recombination^{21,22}. Knockout of the *Terb1* gene in mice disrupts the entire interaction network and impairs homolog pairing, synapsis, and recombination, leading to early abolishment of both spermatogenesis and oogenesis²¹. However, the significance of each of the TERB1-mediated interactions and their molecular mechanisms in meiosis remain unclear.

In the present study, we characterized the TERB1-TRF1 interaction and determined the crystal structure of a short TRF1-binding motif of human TERB1 in complex with the TRF-homology (TRFH) domain of human TRF1. Our structural and biochemical characterization reveals that TRF1 recognizes a unique IxLxP motif on TERB1 via the peptide-binding site

in its TRFH domain. We generated knock-in mice with the TRF1-binding-deficient mutation in the *Terb1* gene and studied the functional roles of the TERB1-TRF1 interaction in mouse meiosis. Strikingly, specific disruption of the TERB1-TRF1 interaction led to infertility only in male mice. We found that the *Terb1* mutation caused an arrest in the zygotene-early pachytene stage of spermatogenesis and impaired X-Y chromosome pairing, resulting in massive spermatocyte apoptosis. The sex chromosomes possess short PARs²⁵, and X-Y pairing is more challenging than autosomal pairing, owing to the deficiency of DNA interaction along the length of the chromosomes^{26,27}. Our studies suggest a mechanism of telomeres that helps surmount the unique challenges of X-Y pairing in meiosis.

RESULTS

Characterization of the interaction between TERB1 and TRF1

The TRFH domain of TRF1 is known to interact with TERB1, which functions together with TRF1 and SUN1 to regulate telomere-nuclear envelope attachment and telomere movement in meiosis^{20,21}. TRF1_{TRFH} was reported to directly interact with a C-terminal fragment of TERB1 (residues 522–662)²¹ (**Fig. 1a**). We confirmed this interaction using the yeast two-hybrid (Y2H) assay (**Supplementary Fig. 1**). To further map the TRF1-binding region in TERB1, we generated various TERB1 fragments of different lengths within TERB1_{522–662} to evaluate their ability to interact with TRF1_{TRFH} by Y2H analysis. Our data revealed that a short fragment of TERB1 consisting of residues 642–656 was sufficient for binding to TRF1 (**Supplementary Fig. 1**). TERB1_{642–656} binds TRF1_{TRFH} with an equilibrium dissociation constant (K_d) of 5.6 μ M, measured by isothermal titration calorimetry (ITC) assay (**Fig. 1b**). Hereafter, we will refer to TERB1_{642–656} as TERB1_{TBM} (TRF1-binding motif) (**Fig. 1a**). Our previous studies have demonstrated that TRF1_{trfh} specifically recognizes a short peptide motif with a signature sequence FxLxP (x, any amino acid) on its interacting proteins, including TIN2 (ref. 28). TERB1_{TBM} contains a modified sequence FxLxP that highly resembles the TRF1-binding motif of TIN2 (TIN2_{TBM}) (**Fig. 1c**), suggesting that TERB1_{TBM} might bind to TRF1_{trfh} in the same manner as TIN2_{TBM}.

Crystal structure of the TERB1_{TBM}-TRF1_{TRFH} complex

To reveal the structural basis for the specific recognition of TERB1 by TRF1, we crystallized TRF1_{TRFH} in complex with the TERB1_{TBM} peptide and determined the structure at a resolution of 2.1 Å by molecular replacement, using the previously determined structure of TRF1_{TRFH} as a search model²⁸ (**Table 1**, **Fig. 1d** and **Supplementary Fig. 2a**). Except for three residues at the N and C termini, the TERB1_{TBM} peptide is well ordered, as evidenced by good electron density in the final atomic model (**Supplementary Fig. 2a**).

The TRFH domain of TRF1 forms a homodimer, and each subunit contains ten meandering α -helices (**Fig. 1d**). The TERB1_{TBM} peptide binds in an extended conformation across the concave surface of each TRF1_{TRFH} subunit, burying ~ 700 Å² of total surface area at the intermolecular interface (**Fig. 1d**). Each of the TERB1_{TBM} peptides contacts only one TRF1_{TRFH} subunit and does not interfere with the dimerization of TRF1_{TRFH} (**Fig. 1d** and **Supplementary Fig. 2a**). The N terminus (⁶⁴⁵ILLTP⁶⁴⁹) of the TERB1_{TBM} peptide adopts a sharp-turn conformation and binds to TRF1_{trfh} through both van der Waals and electrostatic

interactions (**Fig. 1e**). The side chain of Leu647^{TERB1} is inserted deeply into a hydrophobic pocket formed by a panel of residues of TRF1_{trfh} with hydrophobic and aliphatic side chains (Leu86, Arg102, Ala105, Glu106, Ile109, Ile123, Cys126, Gln127, and Thr130) (**Fig. 1e**). This van der Waals contact is buttressed by an elaborate network of hydrogen bonds between the backbone of TERBITBM surrounding Leu647^{TERB1} and the side chains of TRF1 Glu106, Gln127, Thr130, and Arg131 (**Fig. 1e**). In addition to Leu647^{TERB1}, Pro649^{TERB1} also contributes to complex formation via a stacking interaction between its pyrrolidine ring and the phenyl ring of Phe142^{TRF1} (**Fig. 1e**). The interaction between TRF1_{trfh} and the C-terminal positively charged residues of TERBITBM (⁶⁵⁰RRR⁶⁵²) is dominated by electrostatic contacts (**Fig. 1f** and **Supplementary Fig. 2b**). This segment of TERBITBM adopts a short β -strand conformation that runs antiparallel to the one formed by the conserved residues (¹³⁹DAQ¹⁴¹) of TRF1 (**Fig. 1d,f**). This intermolecular β -sheet conformation is stabilized by electrostatic interactions; the guanidinium groups of Arg652^{TERB1} and Arg650^{TERB1} make four hydrogen-bonding interactions with Leu138, Asn144, and Glu146 of TRF1 (**Fig. 1f**).

The structure of the TERB1_{tbm}-TRF1_{trfh} complex reveals a striking similarity to that of the TIN2_{tbm}-TRF1_{trfh} complex (**Supplementary Fig. 3a-c**). First, the central L-X-P motifs in both TRFH-binding motifs are identical in overall conformation, and the two key hydrophobic residues Leu647 and Pro649 of TERB1 interact with TRF1 in exactly the same way as their counterparts in TIN2_{tbm} do (**Supplementary Fig. 3a-c**). Second, in both complex structures, the side chain of one arginine residue (Arg652 in TERB1 and Arg266 in TIN2) at the C terminus of both TBM motifs points to a concaved acidic surface on TRF1_{trfh} (**Supplementary Fig. 3a-c**), suggesting that these electrostatic contacts make important contributions to the interaction between TRF1 and its binding partners. The major difference between the two structures is at the N termini before the central L-X-P motifs of the TBM peptides: ⁶⁴⁴KIL⁶⁴⁶ of TERB1 folds back onto the aliphatic side chain of Arg650^{TERB1} to form a closed configuration, whereas ²⁵⁷HFN²⁵⁹ of TIN2 adopts a rather extended conformation with the phenyl ring of Phe258^{TIN2} sitting on a flat hydrophobic surface of TRF1_{trfh} (**Supplementary Fig. 3a-c**).

Mutational analysis of the TERB1-TRF1 interface

To test the validity of the TERB1-TRF1 interaction observed in the crystal structure, we mutated residues of the TERB1_{tbm} Peptide that were predicted to be important for interacting with TRF1 and then examined binding. We first focused on two highly conserved hydrophobic residues in TERB1, Leu647 and Pro649, whose equivalents in other TRFH-binding proteins are critical for their respective interactions^{28–30}. Consistent with the structural data, ITC analysis with purified TRF1_{trfh} and TERB1_{tbm} peptides showed that mutations of these key interacting residues either completely abolished (TERB1^{L647A}) or substantially impaired (TERB1^{P649A}) the TERB1-TRF1 interaction (**Supplementary Fig. 4**). A previous study showed that TERB1^{T648} can be phosphorylated by cyclin-dependent kinase and that this modification is not compatible with the interaction between TERB1 and TRF1 (ref. 22). Indeed, a TERB1_{tbm} peptide with a phosphorylated Thr648 or a phosphomimetic glutamate residue at the position of Thr648 completely disrupted the interaction between TERB1_{tbm} and TRF1_{TRFH} (**Supplementary Fig. 4**). This finding is consistent with the crystal structure in which the hydroxyl group of TERB1^{T648} is

surrounded by a negative electrostatic surface of TRF1, which would generate unfavorable electrostatic conflict with TERB1 if a phosphorylated threonine or a glutamate residue were at the position of TERB1^{T648}(**Supplementary Fig. 2b**).

We then tested the interactions of mutant proteins transiently expressed in human embryonic kidney 293T cells, and the co-immunoprecipitation (co-IP) results were consistent with the *in vitro* ITC measurements (**Fig. 2a**). We therefore conclude that the observed TERB1-TRF1 interface is specific and necessary for both *in vitro* and *in vivo* binding of TERB1 to TRF1. Next, we tested the relevance of the TERB1-TRF1 interaction to the telomeric localization of TERB1 in HeLa cells by transiently expressing Flag-tagged WT or mutant TERB1. Indirect immunofluorescence (IF) analysis revealed a nuclear punctate staining pattern for WT TERB1 that almost completely colocalized with TRF1 and telomeres (**Fig. 2b**). In contrast, TERB1 mutants containing single or multiple point mutations that disrupt the TRF1-TERB1 interaction were distributed throughout the nucleoplasm with no obvious accumulation at telomeres (**Fig. 2b**). This result confirms the structural information and indicates that the binding of TERB1_{IBM} to the TRFH domain of TRF1 is required for the telomeric localization of TERB1.

Disruption of the TERB1-TRF1 interaction results in decreased fertility in male mice

To address the functional significance of the TERB1-TRF1 interaction in mammalian meiosis, we generated knock-in mice with the TRF 1-binding-deficient mutation ⁶⁴⁷LTP⁶⁴⁹ to ⁶⁴⁷AEA⁶⁴⁹ in the *Terb1* gene using the CRISPR-Cas9 method³¹ (**Supplementary Fig. 5a,b**). The heterozygous *Terb1*^{LTP/AEA} mice were healthy and fertile and were intercrossed to produce homozygous *Terb1*^{AEA/AEA} mice. Pups were born close to the expected Mendelian ratio, and homozygous *Terb1*^{AEA/AEA} mice exhibited similar body weight compared to their WT and heterozygous littermates (data not shown), suggesting that the *Terb1*^{AEA/AEA} mutation does not impair embryonic development. Quantitative PCR (qPCR) and western blotting with mouse testis revealed that the *Terb1*^{AEA/AEA} mutation did not alter the *in vivo* mRNA and protein expression level of TERB1 (**Supplementary Fig. 5c,d**). Strikingly, although some *Terb1*^{AEA/AEA} males produced offspring, most were infertile. In sharp contrast, *Terb1*^{AEA/AEA} females were fully fertile with normal litter sizes, strongly suggesting that the ⁶⁴⁷AEA⁶⁴⁹ mutation of TERB1 only affects male fertility.

Six-week-old testes of *Terb1*^{AEA/AEA} male mice were hypoplastic and about 35% in size compared with those of WT and heterozygous mice (**Fig. 3a**). Histopathological analysis showed that the seminiferous tubules were narrower and lacked postmeiotic cells (**Fig. 3b**). Furthermore, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis of testis sections showed numerous apoptotic signals in *Terb1*^{AEA/AEA} seminiferous tubules, indicating that spermatocytes were massively eliminated in *Terb1*^{AEA/AEA} testes (**Fig. 3c,d**). Consequently, there were no or very few mature sperms in either the caput or cauda epididymal lumen of *Terb1*^{AEA/AEA} testes (**Fig. 3b**). In sharp contrast, both the size of ovaries and the number of primordial follicles were indistinguishable between *Terb1*^{AEA/AEA} and control females (**Fig. 3e,f**), in accordance with the observation that *Terb1*^{AEA/AEA} females were fully fertile.

Disruption of the TERB1-TRF1 interaction causes progressive loss of advanced spermatogenic cells

To reveal the reason for the absence of spermatozoa in *Terb1^{AEA/AEA}* mice, we first analyzed meiotic progression using fluorescence-activated cell sorting (FACS) analysis. We found that although mature haploid sperms were generated in WT and heterozygous mice, the spermatogenesis process in *Terb1^{AEA/AEA}* mice was disrupted at the tetraploid stage of primary spermatocytes (**Fig. 4a,b**). We further examined the progression of meiotic prophase I by immunostaining the axial element component SYCP3 and the synaptonemal complex protein SYCP1 on spermatocyte spreads^{32,33}. In WT mice at 21 d postpartum, more than 60% of the spermatocytes that expressed SYCP3 reached the pachytene or later stages (**Fig. 4c,d**). In contrast, in *Terb1^{AEA/AEA}* mice, most of the spermatocytes were arrested in the zygotene (~60%) or pachytene-like (~15%) stages, and very few spermatocytes reached the diplotene stage (**Fig. 4c,d**). Consistent with the immunostaining analysis, the Hoechst 33342 staining profile of *Terb1^{AEA/AEA}* spermatocytes showed an arrest at the zygotene-early pachytene stage (**Supplementary Fig. 6a-c**). Next, we used fluorescence *in situ* hybridization (FISH) analysis of testis sections to further investigate the meiotic defect of *Terb1^{AEA/AEA}* mice. There were very few spermatocytes exhibiting telomere bouquets in WT and heterozygous testis sections, consistent with the fact that telomere clustering in the bouquet configuration occurs transiently during the zygotene stage^{34–36} (**Fig. 4e,f**). In contrast, there was an ~4-fold increase of the frequency of nuclei with telomere bouquets in *Terb1^{AEA/AEA}* testis sections (**Fig. 4e,f**). Collectively, these results suggest that spermatogenesis in *Terb1^{AEA/AEA}* mice is delayed or arrested predominantly at the zygotene-early pachytene stage.

Given that TERB1 plays an important role in homologous pairing, synapsis, and recombination during meiosis²¹, we next examined whether *Terb1^{AEA/AEA}* spermatocytes exhibit defects in these processes. IF staining of SYCP3, SYCP1, and MLH1 showed that autosomal homologous pairing, synapsis, and MLH1 focus formation (a crossover marker) appeared normal in *Terb1^{AEA/AEA}* spermatocytes (**Fig. 4c** and **Supplementary Fig. 7a,b**). To analyze whether the repair of meiotic programmed DSBs was impaired in *Terb1^{AEA/AEA}* testes, we analyzed spermatocyte spreads using γ -H2AX as a marker of DSBs³⁷. WT and *Terb1^{AEA/AEA}* spermatocytes exhibited a similar γ -H2AX staining pattern with γ -H2AX distributed throughout entire nuclei in leptotene and early zygotene, solely restricted to the sex body in midpachytene to late diplotene, and eliminated in diakinesis (**Supplementary Fig. 7c**). Taken together, our data suggest that, although the spermatogenesis is delayed in the zygotene-early pachytene stage, autosomal homologous crossing over, pairing, and synapsis are largely not affected in *Terb1^{AEA/AEA}* mice.

Disruption of the TERB1-TRF1 interaction impairs telomeric localization of TERB1 and SUN1

We demonstrated that point mutations of any residue in ⁶⁴⁷LTP⁶⁴⁹ of TERB1 disrupted the interaction between TERB1 and TRF1 when both proteins were tagged and coexpressed in HeLa cells (**Fig. 2a** and **Supplementary Fig. 4**). We next examined the effect of the ⁶⁴⁷AEA⁶⁴⁹ mutation of TERB1 in the interaction between endogenous TERB1 and TRF1 in *Terb1^{AEA/AEA}* spermatocytes. Co-IP experiments showed that the ⁶⁴⁷AEA⁶⁴⁹ mutation greatly weakened the interaction (**Supplementary Fig. 8a**). This result confirmed that the

TBM motif of TERBI is essential for TERB1-TRF1 interaction in spermatocytes. We then investigated whether this mutation causes any defect in telomere localization of TERBI in *Terb1^{AEA/AEA}* spermatocytes. IF analysis of WT spermatocyte spreads showed discrete TERB1 foci completely colocalized with telomeres (**Fig. 5a**). In contrast, most telomeres exhibit substantially reduced TERB1 staining, and some of the telomeres completely lack TERB1 signal in *Terb1^{AEA/AEA}* spreads (**Fig. 5a,b**). IF staining of SUN1 showed a similar defect in telomere localization in *Terb1^{AEA/AEA}* spreads (**Supplementary Fig. 8b,c**), consistent with previous data that TERB1 recruits telomeres to INM through simultaneous interactions with both TRF1 and SUN1 in mouse spermatocytes²¹. Therefore, we conclude that disruption of the TERB1-TRF1 interaction impairs telomeric localization of TERBI and SUN1 in spermatocytes.

Disruption of the TERB1-TRF1 interaction causes severe defect in sex-chromosome pairing

The telomere localization defect of the TERB1⁶⁴⁷AEA⁶⁴⁹ mutant protein prompted us to investigate whether this mutation could cause structural abnormalities at telomeres such as splitting, bridging, and fusion, which are shown in *Terb1* spermatocytes²¹. Indeed, we observed on average two telomere splits or bridges and 1.5 telomere fusions between autosomal chromosomes per *Terb1^{AEA/AEA}* spread (**Fig. 5c-e**). These aberrant telomeres affected ~10% of autosomal chromosomes, which is > 10-fold more frequent than in WT spermatocytes (**Fig. 5c-e**). Nevertheless, this phenotype is relatively mild and much less pronounced than the telomere aberrations in cells lacking TRF1 or TRF2 (refs. 38,39).

The most notable phenotype in *Terb1^{AEA/AEA}* spermatocytes was a very high incidence (~70%) of unpaired sex chromosomes (X and Y chromosomes) (**Fig. 5f**), similar to the frequency of unpaired sex chromosomes observed in *Spo11β*-only spermatocytes²⁷. In sharp contrast, no asynapsed autosomal chromosomes were found in *Terb1^{AEA/AEA}* spermatocytes (**Fig. 5d,g**). In addition, *Terb1^{AEA/AEA}* spermatocyte spreads also showed telomere fusions between sex and autosomal chromosomes with a frequency much higher than that of fusions between autosomal telomeres (**Fig. 5g**). Taken together, these data reveal that, although the 647AEA⁶⁴⁹ mutation of TERB1 causes telomere abnormalities in both autosomal and sex chromosomes, the defects of sex chromosomes are much more severe than those of autosomes.

DISCUSSION

In this report, we used both X-ray crystallography and mutational analyses to demonstrate that the TRFH domain of TRF1 specifically recognizes a short TBM sequence of TERB1, providing the first structural basis for how the telomere-INM connection is formed in meiosis. We have previously shown that the TRFH domain of TRF1 functions as a docking platform and recognizes a conserved TBM with a signature sequence of FxLxP in telomeric and telomere-associated proteins²⁸. Notably, our data reveal that TERB1_{TBM} contains a modified TBM sequence, FxLxP, and that this motif of TERB1 and proteins that contain TBM sequences, including TIN2, all bind to the same molecular-recognition surface of the TRF1_{Trph} domain in a manner that could be mutually exclusive (**Supplementary Fig. 3a-c**). It is well established that TIN2 occupies a central position in the shelterin complex and

simultaneously interacts with TRF1, TRF2, and TPP1, bridging three major DNA-binding modules in a highly organized manner^{40–42}. The mutually exclusive TRF1 association between TERB1 and TIN2 suggests that there exist two separate TRF1-containing complexes in meiosis: the shelterin complex for telomere capping and the complex containing TERB1, TERB2, and MAJIN for specific telomere functions in meiosis.

TERB1, TERB2, and MAJIN form a meiotic telomere regulatory complex that anchors telomeres at the INM with the LINC complex and meiotic cohesin complex^{21,22}. This meiotic-specific interaction network is dynamically regulated and crucial for chromosome movement and homologous pairing, synapsis, and recombination^{21,22}. Disruption of the TERB1-TRF1 interaction partially abolishes this interaction network and leads to telomere aberrance and spermatogenesis arrest or delay in *Terb1*^{AEA/AEA} mice (**Figs. 4 and 5**). This telomere aberrance is relatively mild, and a substantial amount of *Terb1*^{AEA/AEA} spermatocytes reach the pachytene stage with successful homolog pairing, synapsis, and recombination (**Fig. 4c,d** and **Supplementary Fig. 7a,b**). Unlike autosomes, most of the X and Y chromosomes are unpaired in *Terb1*^{AEA/AEA} pachytene spermatocytes (**Fig. 5f**). X-Y pairing is intrinsically more challenging than autosomal pairing, because X and Y chromosomes have only a very short subtelomeric PAR (<1 Mb) and cannot mediate efficient multiple-DNA interactions along the entire length of the chromosomes. We propose that meiotic-specific telomere structure that is mediated by the TERB1-TRF1 interaction and immediately adjacent to the PAR may play an important role in promoting X-Y pairing. Disruption of the TERB1-TRF1 interaction results in unpaired X and Y chromosomes at pachytene, consequently triggering an apoptosis response and leading to male infertility. In contrast, homologous pairing and synapsis in females can take place along the nearly 170-Mb length of the X chromosome and is therefore unlikely to require special help from telomeres, so that the *Terb1*^{AEA/AEA} female mice are fully fertile. Because the TERB1-TRF1 interface is conserved in humans, we speculate that TRF1-binding-deficient variants of TERB1 may be a human X-Y nondisjunction-susceptibility trait. We propose that aside from the critical capping function, telomeres play a vital role in meiosis via assisting X-Y pairing.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ONLINE METHODS

Protein expression and purification.

Human TRF1_{trph} (residues 65–267) and TERB1_{TBM} (residues 642–656) were cloned into modified pET28a vector with a SUMO protein fused at the N terminus after the 6 x His tag and expressed in *E. coli* BL2-CodonPlus (DE3) cells (Stratagene). After induction for 18 h with 0.1 mM IPTG at 18 °C, the cells were harvested by centrifugation and the pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% Glycerol, 1mM PMSF, 5 mM benzamidine, 1 µg/mL leupeptin and 1 µg/mL pep- statin). The cells were then lysed by sonication and the cell debris was removed by ultracentrifugation. The supernatant

was mixed with Ni-NTA agarose beads (Qiagen) and rocked for 1 h at 4 °C before elution with 250 mM imidazole. Then Ulpl protease was added to remove the His-SUMO tag. TRF1_{trfh} protein was further purified by gel-filtration chromatography on HiLoad Superdex75 column (GE Healthcare) equilibrated with 25 mM Tris-HCl, pH 8.0, 150 mM NaCl and 5 mM DTT. The purified TRF1_{TRFH} protein was concentrated to 20 mg/mL and stored at -80 °C. The TERB1_{TBM} peptide was further purified by gel-filtration chromatography on HiLoad Superdex75 column (GE Healthcare) equilibrated with 100 mM ammonium bicarbonate. The purified TERB1_{tbm} was concentrated by Speed Vac system and then lyophilized. The lyophilization products were then resuspended in water at a concentration of 50 mg/mL and stored at -80 °C. WT and mutant TERB1_{TBM} peptides used for ITC measurements were expressed and purified similarly.

Crystallization, data collection, and structure determination.

TRF1_{trfh} and TERB1_{TBM} were mixed at a molar ratio of 1:3 and the mixture was used for crystallization. Crystals of the TRF1_{trfh}-TERB1_{tbm} complex were grown using the sitting drop vapor diffusion method at 16 °C. The precipitant-well solution consisted of 10% 2-propanol, 0.1M Tris-HCl, pH 8.5. Crystals were gradually transferred into a harvesting solution containing 20% 2-propanol, 0.1M Tris-HCl, pH 8.5, and 25% glycerol before being flash frozen in liquid nitrogen for storage. Data sets were collected under cryogenic conditions (100 K) at the Shanghai Synchrotron Radiation Facility (SSRF) beamlines BL18U1 and BL19U1 (wavelength 0.97853 Å) and were processed by HKL3000 (ref. 43). TRF1_{trfh}-TERB1_{TBM} complex structure was solved by molecular replacement by using the previously published TRF1_{TRFH}-TIN2_{Tbm} complex structure (PDB 3BQO) as a searching model. The model was then refined using Refmac⁴⁴, together with manual building in Coot⁴⁵. In the final Ramachandran plot, the favored and allowed residues are 96.8% and 100.0%, respectively. All of the crystal structural figures were generated using PyMOL⁴⁶.

Isothermal titration calorimetry (ITC).

The equilibrium dissociation constants of the WT and mutant TERB1_{TBM} interactions with TRF1_{trfh} were determined using a MicroCal iTC200 calorimeter (Malvern). The enthalpies of binding between TRF1_{trfh} (50–100 µM) and TERB1_{TBM} (500–1,000 µM) were measured at 16 °C in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl. Three independent experiments were performed for every interaction described here. ITC data were subsequently analyzed and fit using Origin 7 software (OriginLab) with blank injections of peptides into buffer subtracted from the experimental titrations before data analysis.

Yeast two-hybrid assay.

The yeast two-hybrid assays were performed as described previously⁴⁷. Briefly, the L40 strain was transformed with pBTM16 and PACT2 (Clontech) fusion plasmids, and colonies harboring the both plasmids were selected on -leucine -tryptophan plates. The β-galactosidase activities were measured with a liquid assay.

Animals.

Mice were housed under controlled environmental conditions with free access to water and food. Experimental protocols were approved by the regional ethical committee of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. We made every effort to minimize and refine our experiments to avoid animal suffering.

Generation of Terbl^{AEA/AEA} mice.

The Terbl^{LTP/LTP} to Terbl^{AEA/AEA} mutation mice were generated using the CRISPR-Cas9 and single strand oligos as a donor³¹. Briefly, a single guide RNA (sgRNA: ACAAAGTTGTCTTCTTCTAC) was cloned into a sgRNA vector using OriGenes gRNA Cloning Services (Rockville, Maryland) and was then used as a template to synthesize sgRNAs using the MEGAshortscript T7 Kit (Life Technologies). Cas9 mRNA was *in vitro* transcribed from plasmid MLM3613 (Addgene #42251) using the mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies). For microinjection, Cas9 mRNA (100 ng/μL) was mixed with sgRNA (50 ng/μL) and 100 ng/μL donor oligos (AAC TAC TTA AAT TAC TGT TTT AAG TAT ACT AAA TGT TTT TTA TAT TTT TCA GAA ATT TTG GCG GAA GCA TGC AGA AGA AGA CAA CTT TGT AAA GAA TCT ACT GCC TCT GAA GAA CTA AGT AAG TAT ATT) and then microinjected into the cytoplasm of fertilized eggs collected from B6CBAF1/J FI hybrid mice (JAX). The injected zygotes were cultured overnight in M16 medium at 37 °C in 5% CO₂. In next morning, the embryos that had reached two-cell stage of development were implanted into the oviducts of pseudo-pregnant foster mothers (Swiss Webster, Taconic Farm). The mice born to the foster mothers were genotyped by tail biopsy. Briefly, purified tail DNA samples were amplified by PCR using a pair of forward (TCT ACA GCC ACC AGG CAC) and reverse (AGG GAG GAA GAC GGT GTC) primers. The PCR bands were then sequenced using primers GTT CAC TA A GGA TTC CTA CTA G (forward orientation) and TCA TGA AAG CCA AGG GTT ATC (reverse orientation). Mice with overlapping sequencing peaks at the intended mutation sites were bred with C57BL/6 mice for passing the desired mutations through the germline.

Histological analysis.

The testes and ovaries were dissected and fixed in 4% paraformaldehyde in PBS for 4–12 h at 4 °C. Dehydration of tissues was done by passage through graded ethanol, and then tissues were cleared twice in xylene and embedded in paraffin. Tissue sections (5 μm) were prepared with a Leica RM2235 rotary microtome. HE staining was performed according to the standard protocol (Beyotime, CO 105). TUNEL assay was carried out with TUNEL Apo- Green Detection Kit (Biotool, B31112).

Quantitative real-time PCR.

Total RNA was purified from mouse testes using Trizol reagent (Invitrogen, 15596026) and total cDNA was prepared from total RNA using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, 6210A). Quantitative real-time PCR was performed with the Power SYBR Green PCR Master Mix (ABI, 4367659) using the ViiA 7 System (Thermo Fisher Scientific). The primers used were as follows: mTerbl-forward: 5'-

TTCGTCACCTGCACAGAGGATTG-3'; mTerbl-reverse: 5'-CACGTAAGTCGCAGCTTTGC-3'; mGAPDH-forward: 5'-GGTGAAGGTCGGTGTGAACG-3'; mGAPDH-reverse: 5'-CTCGCTCCTGGAAGATGGTG-3'

Antibody production.

cDNA fragment encoding mTERB1 (residues 384–565) was inserted into pET28a vector with a His-SUMO tag and was expressed in *E. coli* BL2-CodonPlus (DE3) cells (Stratagene). Recombinant proteins were purified and His-SUMO tag was removed before immunizing rabbits. Polyclonal antibodies were affinity purified on antigen-coupled Sepharose beads (GE Healthcare).

Antibodies.

The following antibodies were used: rabbit polyclonal antibody against TERB1 (this study), SUNT (ref. 19), H2AX (p-S139) (Novus, NB100–384), SYCP1 (Abcam, ab15087), SYCP3 (Abeam, ab15093), TRF1 (ref. 28), c-myc (Santa Cruz, sc-789); mouse monoclonal antibodies against MLH1 (Abcam, ab14206), SYCP3 (Abcam, ab97672), TRF1 (Abcam, ab66223), c-myc (Santa Cruz, sc-40), FLAG (Sigma, F3165), GAPDH (Cmctag, AT0002), actin (Sigma, A2228). Secondary antibody for western blot: Goat anti-Mouse IgG/AP (Beyotime, A0216), Donkey anti-Rabbit IgG/AP (BBI, D110057), Goat anti-Mouse IgG/HRP (ABclonal, AS003), Goat anti-Rabbit IgG/HRP (Proteintech, SA00001). Goat anti-mouse/rabbit secondary antibody for immunofluorescence: DyLight 488 (thermo, 35502, 35553), DyLight 550 (thermo, 84540, 84541), DyLight 633 (thermo, 35512, 35562).

Cell culture.

293T and HeLa cells were obtained from the cell bank of type culture collection of Chinese Academy of Sciences and were cultured at 37 °C under 5% CO₂ in DMEM supplemented with 10% FBS or cosmic calf serum (Hyclone). No mycoplasma contamination was detected in these cells.

HeLa stable cell lines.

Full-length hTERB1 WT and mutants were constructed into pLVX-IRES-Puro (Clontech) and transfected into 293T cell with X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manual. Lentiviruses are harvested 40 h later and filtered by 0.22 µm filter (Millipore, SLGP033R) before infecting plain HeLa cells. 48 h after infection, 1 µg/mL puromycin was added to establish the stable-expressing populations. After 5 d of selection, cells were ready for immunofluorescence staining.

Co-IP and western blot.

cDNA fragments were constructed into pEGFP-N1 (Clontech) and transfected into 293T cells. After 36 h, cells were collected and lysed and sonicated in lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% Glycerol, 0.5% TritonX-100, 1 mM EDTA) supplemented with EDTA-free complete protease inhibitor (Roche), sonicated on ice, and centrifuged (13,000 r.p.m., 15 min, 4 °C). The resulting supernatants were then precleared and immu-

noprecipitated with anti-FLAG M2 Affinity Gel (Sigma) overnight at 4 °C with constant rotation. Precipitates were then washed with lysis buffer, resuspended in Laemmli buffer, boiled for 5 min and used immediately on SDS-PAGE for immunoblotting. To detect WT and mutant TERB1 proteins in spermatocytes, testis samples from mouse littermates were homogenized in PBS buffer. Cells were pelleted and then lysed and sonicated in lysis buffer. Lysates were centrifuged and supernatants were prepared for SDS-PAGE. To detect the interaction between endogenous TERB1 and TRF1 in spermatocytes, mouse testis cell lysates were immunoprecipitated with anti-TERB1, and immunoprecipitated samples were subjected to western blot analysis. After SDS-PAGE, proteins were blotted onto PVDF membranes (Millipore). The blots were incubated in blocking buffer (5% fat-free milk in PBS buffer supplemented with 0.05% TWEEN-20) for 1 h at RT and were incubated in primary antibodies overnight at 4 °C. Blots were then washed and incubated in the alkaline-phosphatase-labeled or HRP-labeled secondary antibody for 1 h at RT. After being washed, blots were developed with BCIP/NBT substrate kit (Invitrogen, 002209) or ECL Prime western Blotting System (GE Healthcare, RPN2232).

Hoechst 33342 and PI staining for flow cytometry.

Testes tissues were prepared for FACS based on previous studies^{48,49} with minor modifications. Briefly, decapsulated testes were incubated in DMEM medium supplemented with 1 mg/ml Collagenase type IV (Sigma) and 10 µg/mL DNase I (Roche) in 50-ml tubes at 33 °C for 20 min and shaken vigorously by hand. 1 mg/mL trypsin (Gibco) was then added to the tissue incubation medium and samples were incubated at 33 °C for another 20 min, with the tube being inverted several times every 5 min at 33 °C. The dissociated testis samples were pipetted with a plastic disposable Pasteur pipet ten times, and 10% FBS was added to inactivate trypsin. For Hoechst 33342 staining, samples were incubated with 100 µg/mL Hoechst 33342 and 10 µg/mL DNase I at 33 °C for 1 h and then passed through 40-µm cell strainers (BD Falcon). 10 µg/mL PI was added before FACS processing performed using a BD Influx cell sorter. For PI staining of fixed whole cells, dissociated testis samples were centrifuged (2,000 r.p.m., 15 min, 4 °C) and cell pellets were resuspended in 100 µE PBS and then 900 µE 75% ethanol was added. Cells were fixed overnight at 4 °C and washed once with PBS. Cells were then resuspended in PI staining solution (PBS supplemented with 0.1% TritonX-100, 200 µg/mL DNase-free RNase and 20 µg/mL PI) and incubated at 37 °C for 30min and then used for FACS analysis with a BD FACSCalibur Flow Cytometer.

Spermatocytes chromosome spreading.

Chromosome spreading samples were prepared based on a previous study⁵⁰. Briefly, testes were dissected and placed in PBS. Seminiferous tubules were separated and placed in a hypotonic extraction buffer (30 mM Tris-HCl, pH 8.2, 50 mM sucrose, 17 mM sodium citrate, 5 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail (Roche)) for 30–60 min. Tubules were then moved to 100 mM sucrose solution (pH 8.2) and spermatocytes were detached by pipetting. Drops of cell suspension were then placed on the upper right corner of cover slips (Thermo, 12–545-84) that were soaked in a 1% paraformaldehyde solution (Sigma, P6148), pH 9.2, supplemented with 0.15% Triton X-100. Nuclei were dried overnight at RT and stored at –80 °C.

Immunofluorescence staining, telomere FISH, microscopy, and fluorescence- signal quantification.

For immunofluorescence staining of cultured cells, cells were grown on cover slips (Thermo, T_7011254584) and fixed in 4% paraformaldehyde, permeabilized with 0.15% Triton X-100, blocked with 3% BSA at RT for 1 h and incubated with primary antibodies. For immunofluorescence staining of spermatocyte spreads, spreads were washed with PBS buffer supplemented with 0.15% Triton X-100, blocked with 3% BSA at RT for 1 h and incubated with primary antibodies at 4 °C overnight. For immunofluorescence staining of paraffin-embedded tissue sections, sections on cover slips were dewaxed twice in fresh xylene for 15 min each, rehydrated in 100%, 95%, 75%, 50% ethanol and PBS for 10 min each. Sections were then incubated in retrieval solution (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and boiled for 1 min for antigen retrieval. After washed with PBS at RT for 10 min and blocked with 3% BSA at RT for 1 h, cover slips with sections were incubated with primary antibodies at 4 °C overnight. Cover slips with cells, sections or spreads were then washed and incubated with fluorescence- conjugated secondary antibodies at RT for 1 h. Cover slips were washed and ready for microscopy imaging. Combinatorial immunofluorescence staining and telomere FISH were carried out following the protocol described previously with minor modifications⁵¹. Briefly, after being incubated with secondary antibodies, cover slips were washed and refixed with 4% paraformaldehyde for 10 min, dehydrated in 70%, 85%, and 100% ethanol, air dried, and hybridized to PNA telomere probe (Panagene) in hybridization buffer (20 mM Tris- HCl, pH 7.4, 70% formamide, 0.5% blocking reagent (Roche 11096176001)) using ThermoBrite system (Leica) (denatured at 85 °C for 5 min then kept at RT overnight for hybridization). Cover slips were washed once with hybridization buffer and then washed twice with 2x SSC buffer. Cover slips were then dehydrated in ethanol series and DNA was counterstained with DAPI. Microscopy imaging was then performed by LSM 710 (Zeiss) using 63×NA /1.40 oil. Fluorescence intensity quantification was carried out by using Imaris Image Analysis Software.

Data availability.

The atomic coordinates and structure factors for the TRF1_{trfh}-TERB1_{TBm} complex has been deposited to the Protein Data Bank (PDB) under the accession code PDB 5XUP. Source data for biochemical experiments that support the findings of this study are available from the corresponding author upon reasonable request. A **Life Science Reporting Summary** for this article is available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structural studies of the interaction between TRF1_{trfh} and TERB1_{TBM} (a) Domain organization of TERB1 and TRF1. TRFH domain of TRF1 and TBM of TERB1 are colored in dark blue and yellow, respectively. Shading indicates the interaction between TRF1 and TERB1. ARM, armadillo repeats; CC, coiled coil; Myb, myeloblastosis domain; AM, acidic motif; TRFH, TRF homology domain, (b) ITC measurement of the interaction between TRF1_{trfh} (residues 65–267) and TERB1_{tbm} (residues 642–656), with K_d and error of the fit indicated, (c) Sequence alignment of the TBM of TIN2 and TERB1. *Homo sapiens*, NP_001129977.1; *Pan troglodytes*, XP_001159574.2; *Macaca mulatta*, XP_014981824.1; *Mus musculus*, NP_851289.2; *Rattus norvegicus*, XP_006255118.1; *Canis lupus*, XP_005620927.1; *Bos taurus*, XP_005195308.1; *Ovis aries*, XP_014955952.1; *Gallus gallus*, XP_001232198.3; *Xenopus laevis*, XP_018096997.1; *Xenopus tropicalis*, XP_002933719.2; human TIN2, NP_001092744.1. (d) Ribbon diagram of two orthogonal views of the TRF1_{TRFH}-TERB1_{TBM} complex. TRF1_{trfh} is colored in dark blue and cyan, and TERB1_{tbm} is in yellow and pink. (e, f) The TRF1_{TRFH}-TERB1_{TBM} interface. The color scheme is the same as in d. Residues important for the interaction are shown as stick models. Salt bridges and hydrogen-bonding interactions are shown as magenta dashed lines.

Figure 2. Mutational analysis of the TERB1-TRF1 interaction. **(a)** Co-IP of TRF1 with WT or mutant Flag-tagged TERB 1 in 293T cells. The levels of each protein in the input and IP samples were analyzed by immunoblotting with the indicated antibodies. “Input” contains 5% of the input whole-cell lysate used for IPs. The numbers indicated below the blot images represent the relative signals of co-IPed Myc-TRF1 normalized by the signals of anti-Flag. Band intensity of blots was quantified by the ImageJ software. The signal for WT sample was set as 1. Uncropped blot images are in **Supplementary Data Set 1**. **(b)** Telomere colocalization analysis of TRF1 with WT and mutant TERB1 in HeLa cells. Cells were immunostained with anti-Flag (green) and anti-TRF1 (red), hybridized with the Cy3-TelC probe (purple) to detect telomeres, and stained with DAPI (blue) for nuclei. Scale bar, 5 μ m.

Figure 3.

Disruption of the TRF1-TERB1 interaction results in failure of spermatogenesis. (a) Testes from 6-week-old WT, heterozygous (LTP/AEA) and *Terb1*^{AEAIAEA} (AEA/AEA) mice, (b) Hematoxylin-and-eosin-stained histological cross-sections of testes, caput epididymis, and cauda epididymis from adult mice. White arrows indicate abnormal spermatocyte-like cells in *Terb1*^{AEAIAEA} mice. Asterisks show vacuolated seminiferous tubules in which both spermatozoa and spermatids are absent. Scale bar, 50 μ m. (c) TUNEL assay using testis sections from 6–8-week-old mice. DNA was stained with DAPI (blue), and apoptosis was detected by immunofluorescence TUNEL (green). Many TUNEL-positive cells are observed in *Terb1*^{AEAIAEA} testes. Scale bar, 50 μ m. (d) Number of TUNEL-positive cells per tube, shown as mean and s.d., $n = 3$ mice of each genotype. Testis section samples were subjected to TUNEL assay, and more than 300 seminiferous tubules were assessed for each mouse, (e) Ovaries from 12-week-old WT, heterozygous and AEA/AEA female mice. (f) Hematoxylin-and-eosin-stained ovary sections from WT, heterozygous and *Terb1*^{AEAIAEA} mice. Arrows indicate follicles and asterisks show corpora lutea. Scale bar, 200 μ m.

Figure 4.

Spermatogenesis process is arrested in *Terb1^{AEAlAEA}* spermatocytes. **(a)** Propidium iodide (PI) fluorescence flow analysis of testicular cells from 6-week-old WT, heterozygous and *Terb1^{AEAlAEA}* littermates. Results shown are from one out of three independent experiments showing similar results. 1N, haploid; 2N, diploid; 4N, tetraploid. **(b)** Quantification of subpopulations of **a**. Data are mean and s.d. for $n = 3$ mice of each genotype. One male mouse of each genotype from the same litter was used in each experiment. **(c)** Heterozygous and *Terb1^{AEAlAEA}* spermatocytes stained for SYCP1 (red), SYCP3 (green) and DAPI (blue). Scale bar, 10 μm. **(d)** Quantification of the frequencies of meiotic stages shown in **c**. Numbers of spreads used for quantification are shown. **(e)** Representative images for immunofluorescence FISH assay using testis sections from 14-day-old mice stained for SYCP3 (green) and telomeres (telomeric PNAtIC, red). Arrows indicate clustered telomere signals. Scale bar, 10 μm. **(f)** Quantification of the frequencies of cells with telomere bouquet signals. Data show mean and s.d. of $n = 3$ independent experiments with different sets of mouse littermates. One male mouse of each genotype from the same litter was used in each experiment. Statistical significances (***) were assessed by two-tailed t tests.

Figure 5.

The $647^{AEA}649$ mutation of TERB1 results in telomere localization defect of TERB1, autosomal telomere aberrance and unpaired X and Y chromosomes, (a) Localization of TERB1 (red) and SYCP3 (green) on pachytene spermatocyte spreads from WT and *Terb1*^{AEAJAEA} testes. Scale bar, 10 μ m. (b) Quantification of the intensity of TERB1 foci at chromosome termini. More than ten spermatocyte spreads were used for quantification for each mouse, a.u., arbitrary units. Results are representative of three independent experiments with 6-week-old littermates. One male mouse of each genotype from the same litter was used in each experiment. (c) Representative mid-pachytene spermatocyte spreads from WT and heterozygous mice stained for SYCP3 (green), telomere (teIC, red) and γ -H2AX (purple). Scale bar, 10 μ m. (d-g) Representative images showing chromosome structural abnormalities in *Terb1*^{AEAJAEA} mid-pachytene spermatocyte spreads: telomere splitting and bridging on autosomes (d), autosome-autosome telomere fusions (e), X-Y separation (f), and sex chromosome-autosome telomere fusions (g). Three independent experiments with different sets of mouse littermates were performed with similar results. One male mouse of each genotype from the same litter was used in each experiment. More than 50 spreads were counted for each mouse. Scale bars, 10 μ m. Graphs show quantification of structural abnormalities as mean and s.d. of $n = 3$ independent experiments.

Table 1

Data collection and refinement statistics

TRF1 _{trf} -TERB1 _{tbm} (PDB 5XUP) ^a	
Data collection	
Space group	<i>P</i> 6 ₄
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	161.58, 161.58, 45.93
α , β , γ (°)	90.00, 90.00, 120.00
Resolution (Å)	50.00–2.10 (2.18–2.10) ^b
<i>R</i> _{merge} (%)	12.1 (75.5)
<i>I</i> / σ (<i>I</i>)	14.50 (2.00)
<i>CC</i> _{1/2}	0.99 (0.83)
Completeness (%)	100.0 (100.0)
Redundancy	10.0 (9.8)
Refinement	
Resolution (Å)	46.69–2.10 (2.16–2.10)
No. reflections	40,329
<i>R</i> _{work} / <i>R</i> _{free} (%)	17.68/22.07
No. atoms	
Protein	3,357
Water	152
B factors (Å ²)	
Protein	44.82
Water	46.33
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	0.985

^a A single data set was scaled and merged for the crystal structure.

^b Values in parentheses are for highest-resolution shell.