Speedy A–Cdk2 binding mediates initial telomere–nuclear envelope attachment during meiotic prophase I independent of Cdk2 activation

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Contributed by Jan-Åke Gustafsson, November 16, 2016 (sent for review September 12, 2016; reviewed by Heng-Yu Fan and Hengbin Wang)

Telomere attachment to the nuclear envelope (NE) is a prerequisite for chromosome movement during meiotic prophase I that is required for pairing of homologous chromosomes, synopsis, and homologous recombination. Here we show that Speedy A, a noncanonical activator of cyclin-dependent kinases (Cdks), is specifically localized to telomeres in prophase I male and female germ cells in mice, and plays an essential role in the telomere–NE attachment. Deletion of Spdy in mice disrupts telomere–NE attachment, and this impairs homologous pairing and synopsis and leads to zygote arrest in male and female germ cells. In addition, we have identified a telomere localization domain on Speedy A covering the distal N terminus and the Cdk2-binding Ringo domain, and this domain is essential for the localization of Speedy A to telomeres. Furthermore, we found that the binding of Cdk2 to Speedy A is indispensable for Cdk2’s localization on telomeres, suggesting that Speedy A and Cdk2 might be the initial components that are recruited to the NE for forming the meiotic telomere complex. However, Speedy A–Cdk2-mediated telomere–NE attachment is independent of Cdk2 activation. Our results thus indicate that Speedy A and Cdk2 might mediate the initial telomere–NE attachment for the efficient assembly of the telomere complex that is essential for meiotic prophase I progression.

In mammals, the progression of meiotic prophase I is largely dependent on the dynamic movement of chromosomes along the nuclear envelope (NE) (1, 2). A prerequisite for faithful chromosome movement is the anchoring of telomeres to the transmembrane LINC (linker of nucleoskeleton and cytoskeleton) complex that bridges chromatin to the cytoskeleton (3).

In recent years, several meiosis-specific structural molecules that mediate telomere–NE attachment have been identified in mice, such as TERB1 (telomere repeat binding bouquet formation protein 1), SUN1 (Sad1 and UNC84 domain containing 1), KASH5 (Klarsicht/ANC-I/Syne/homology 5), TERB2 (telomere repeat binding bouquet formation protein 2), and MAJIN (membrane-anchored junction protein), and mice lacking any one of SUN1, KASH5, TERB1, TERB2, or MAJIN display impaired telomere attachment and are sterile (4–8). Moreover, cyclin-dependent kinase 2 (Cdk2) is localized to telomeres in mouse spermatocytes and prophase I oocytes (9), and loss of Cdk2 leads to sterility in both male and female mice (10, 11).

Speedy/RINGO (Rapid inducer of G2/M progression in oocytes) proteins are atypical noncyclin Cdk activators that were first discovered in Xenopus laevis as proteins that induce G2/M transition during oocyte maturation (12, 13). Multiple members of the Speedy family have since been identified in mammals (14–16). Speedy proteins activate Cdks independently of cyclins, and they are characterized by their highly conserved, ∼140-aa central Cdk-binding core, called the Ringo domain (14, 17). In mice, four homologs of Speedy have been identified: Speedy A, Speedy B1a, Speedy B1b, and Speedy B3 (14, 16, 17). Both mouse Speedy A and the human homolog Spy1 are able to induce meiotic recombination when injected into Xenopus oocytes (14, 17). However, the in vivo physiological role of mammalian Speedy A is unknown.

In the present study, we generated Spdy knockout mice and studied the functional roles of Speedy A in mammals. We found that distinct from Xenopus, Speedy A in mice is not involved in oocyte maturation. Instead, Speedy A is only specifically expressed in male and female germ cells at meiotic prophase I, and it is localized to the telomeres. Loss of Speedy A in mice impairs telomere–NE attachment in early meiosis, perturbs homologous recombination, and leads to infertility in both sexes. Moreover,

Significance

In meiotic prophase I, telomere attachment to the nuclear envelope is a prerequisite for subsequent prophase events, such as homologous pairing and recombination. In this study, we show that Speedy A, a noncanonical activator of cyclin-dependent kinases (Cdks), is essential for telomere attachment to the nuclear envelope in mice. We have identified a telomere localization domain in Speedy A, which covers the protein’s distal N-terminus and Cdk2-binding Ringo domain but excludes its Cdk-activation domain. Furthermore, we found that the binding of Cdk2 to Speedy A is essential for Cdk2’s localization to telomeres. Our results suggest that Speedy A–Cdk2 binding might mediate the initial assembly of the meiotic telomere complex, a process that is independent of Cdk2 activation.


Reviews: H.-Y.F., Zhejiang University; and H.W., University of Alabama at Birmingham. The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1618465114/-/DCSupplemental.
we found that the proper attachment of telomeres to the NE is dependent on a telomere localization domain (TLD) on Speedy A, as defined by this study, whereas the C terminus of Speedy A, which is required for Cdk2 activation, is dispensable for the attachment of telomeres to the NE. Furthermore, we showed that the binding of Cdk2 to Speedy A is required for Cdk2 to localize onto telomeres. These results suggest that the function of Speedy A goes beyond that of a noncanonical activator of Cdk as previously suggested (14, 15, 18, 19). Rather, the binding between Speedy A and Cdk2 might mediate the initial assembly of the telomere–NE complex that is essential for meiotic prophase I progression.

Results


diplotene stage were also seen during prophase I in female germ cells (Fig. S1 J–O, arrows).

**Knockout of Speedy A Leads to Loss of Male and Female Germ Cells.**

To study the functional roles of Speedy A in vivo in the female germ line, we generated Spdyaflox/flox mice (Fig. S2). In contrast to findings in Xenopus (12, 13), tissue-specific deletion of SpdyA in postnatal mouse oocytes using Zp3-Cre (23, 24) did not lead to any changes in oocyte maturation (Fig. S3 A–D) or female fertility (Fig. S3E), indicating that Speedy A is dispensable for oocyte maturation in mice.

However, in conventional SpdyA−/− mice (as validated in Fig. 3A), we observed infertility in both males and females. These mice exhibited atrophic gonads (Fig. 3 B and C), and further histological analysis revealed that—in sharp contrast to normal spermatogenesis in SpdyA+/+ males at PD7, PD18, and PD75 (Fig. 3 D, F, and H, arrowheads)—SpdyA−/− males had normal tests at PD7 (Fig. 3E) but displayed a loss of differentiated spermatocytes at PD18 (Fig. 3G, arrowhead) and lacked round and elongated spermatids at PD75 (Fig. 3I, arrowhead).

In females, no apparent difference in oocyte number was observed between SpdyA+/+ (Fig. 3J, arrow) and SpdyA−/− (Fig. 3K, arrow) ovaries at 17.5 dpc. However, compared with the SpdyA+/+ ovary (Fig. 3L and N, arrows), a reduction in oocytes was observed in the SpdyA−/− ovary at PD1 (Fig. 3M, arrow), which led to a complete loss of oocytes by PD5 (Fig. 3O, arrow).

**Telomere–NE Attachment Was Impaired in SpdyA−/− Spermatocytes Leading to Meiotic Arrest Before Telomere Cap Exchange.** To investigate the reasons for germ cell loss during prophase I in SpdyA−/− mice, we analyzed telomere distribution patterns in structurally preserved spermatocytes by staining for TRF1 and synaptonemal complex protein 3 (SYCP3). In leptotene and zygotene cells, most of the TRF1 signal was localized to NE in SpdyA+/+ spermatocytes (Fig. 4 A and C, arrows). In contrast, in SpdyA−/− spermatocytes a considerable proportion of the TRF1 signal was found to be within the nuclei (Fig. 4 B and D, arrowheads). The telomere attachment defect was similar in SpdyA−/− female germ cells; although all telomeres were on the NE in SpdyA+/+ female germ cells (Fig. 4F, arrows), in SpdyA−/− cells several telomeres were observed inside the nucleus (Fig. 4F, arrowheads), and the chromosome morphology showed that these cells did not progress beyond the zygotene stage (Fig. 4F).

In SpdyA−/− spermatocytes at the pachytene stage, the central element SYCP1 (synaptonemal complex protein 1) was found along autosomal chromosomes (Fig. S4A, arrow), the DNA double-strand

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**Fig. 2.** Speedy A is localized to telomeres in structurally preserved spermatocytes during meiotic prophase I. Immunofluorescent staining of structurally preserved PD18 wild-type spermatocytes. Telomeres were stained with TRF1. (A–C) In preleptotene cells, Speedy A was only detected on telomeres that were on the NE (arrows). Telomeres inside the nucleus lacked the Speedy A signal (arrowheads). (D–L) In leptotene, zygotene, and pachytene cells, Speedy A and TRF1 signals overlapped on telomeres (arrows).

**Fig. 3.** Loss of Speedy A leads to depletion of male and female germ cells. (A) Western blot showing that Speedy A was deleted in PD25 testes. β-Actin was used as the loading control, and 40-μg lysate from PD25 SpdyA+/+ and SpdyA−/− testes was loaded in each lane. (B) Ovaries from PD35 SpdyA+/+ and SpdyA−/− female mice. (C) Testes from PD35 SpdyA+/+ and SpdyA−/− male mice. (D and E) At PD7, the number of spermatogonia was comparable between SpdyA+/+ and SpdyA−/− testes as indicated by DDX4 staining. (F and G) By PD18, the number of spermatogonia was comparable between SpdyA+/+ (F) and SpdyA−/− (G) testes, but some seminiferous tubules showed depletion of spermatocytes in SpdyA−/− testes (G, arrowhead). (H and I) By PD75, most of the SpdyA−/− seminiferous tubules were depleted of spermatocytes (arrowhead). (J and K) At 17.5 dpc, the number of oocytes was comparable between SpdyA+/+ (J) and SpdyA−/− ovaries (K). (L and M) The number of oocytes was much lower in the SpdyA−/− ovary (M) compared with the SpdyA+/+ ovary at PD1 (L). (N and O) At PD5, the SpdyA−/− ovaries were depleted of oocytes (O, arrow) compared with the SpdyA+/+ ovary (N).
break (DSB) marker γH2AX was restricted to the unsynapsed regions of the XY body (Fig. S4C, arrow), and the chiasma marker MutL homolog 1 (MLH1) appeared on recombination foci (Fig. S4E, arrow) (25–27). In contrast, in zygotene-like Spdya<sup>−/−</sup> spermatocytes, SYCP1 was partially loaded along the synaptonemal axes (Fig. S4B, arrowhead), γH2AX remained on the chromosomes (Fig. S4D, arrowheads), and MLH1 foci were absent (Fig. S4F, arrowhead). These data suggest that homologous synopsis, DSB repair, and homologous recombination are largely abolished in Spdya<sup>−/−</sup> spermatocytes.

We further analyzed the telomeric cap exchange that takes place in pachytene-stage germ cells (5). In wild-type spermatocytes, we observed that TRF2 (telomeric repeat-binding factor 2), a shelterin protein (28, 29), was disassociated from the end of the chromosome axis and formed a ring structure, indicating that cap exchange had occurred (Fig. 4 G and H, arrows and Inset). However, TRF2 did not form a ring structure in Spdya<sup>−/−</sup> cells (Fig. 4 I and J, arrowheads), suggesting that the cap-exchange process was abolished in the mutant. These data further confirmed that Spdya<sup>−/−</sup> spermatocytes did not progress beyond the pachytene stage.

In both Spdya<sup>+/+</sup> and Spdya<sup>−/−</sup> pachytene cells, TERB1 and MAJIN could be observed on a few telomeres that were attached to the NE (Fig. S4 G–J, arrows). However, the LINC complex component SUN1, which is localized to telomeres in Spdya<sup>+/+</sup> spermatocytes (Fig. S4K, arrow), was observed as a polarized cap along the NE in Spdya<sup>−/−</sup> spermatocytes (Fig. S4L, arrow). These results confirmed that Speedy A is essential for tethering telomeres to the NE during prophase.

**Speedy A–Cdk2 Binding Facilitates the Initial Formation of the Telomere Complex Independent of Cdk2 Activation.** Loss of Speedy A prevented the increase of p39 Cdk2 in PD17 Spdya<sup>+/−</sup> testes (Fig. S5A), and the kinase activity of Cdk2 was reduced in Spdya<sup>−/−</sup> testes extracts (Fig. S5B). By immunoprecipitation, we showed that Speedy A indeed interacts with Cdk2 in the testis (Fig. SSC). Moreover, using superresolution microscopy, we found that during meiotic prophase I, Speedy A appears on the NE before Cdk2, implying that Speedy A might recruit Cdk2 to the NE (Fig. S5 D–O). Furthermore, staining of Spdya<sup>−/−</sup> leptotene cells showed that Cdk2 was not localized to telomeres in the absence of Speedy A (Fig. S5 P–R, arrowheads).

In vitro studies have suggested that Speedy A’s Ringo domain (amino acids 67–199) is essential for Cdk2 binding and that its C terminus (amino acids 200–310) promotes Cdk2 activation, but its N terminus (amino acids 1–66) has no known function (14). Using a kinase assay, we confirmed that the C terminus of Speedy A is essential for Cdk2 activation (Fig. S6).

To study how Speedy A is localized to telomeres, we expressed GFP-tagged fragments of Speedy A protein in wild-type mouse testes by electroporation (30) (illustrated in Fig. 5A). Western blot results demonstrated that these Speedy A protein fragments were correctly expressed, as shown in 293T cells (Fig. S7A, asterisks). Full-length Speedy A protein (amino acids 1–310) was localized to telomeres (Fig. S7 B–D, arrows). Moreover, the Speedy A fragment lacking the C terminus (amino acids 1–199) (Fig. S7 E–G, arrows) and the fragment consisting of the Ringo domain plus 3 aa of the N terminus (amino acids 64–199) (Fig. S7 K–M, arrows) could also localize to telomeres. In contrast, the fragment consisting of only the Ringo domain (amino acids 67–199) (Fig. S7 H–J, arrows) failed to localize to telomeres. These results suggested that the amino acids 64–199 region of Speedy A is a prerequisite for Speedy A to localize to telomeres, whereas the Cdk-activating C terminus (amino acids 200–310) is dispensable for the binding of Speedy A onto telomeres.

We next electroporated the GFP-tagged Speedy A fragments into live Spdya<sup>−/−</sup> testes. The full-length Speedy A protein (amino acids 1–310) rescued the telomere attachment defect as characterized by fully recovered TRF1 signal on the NE in structurally preserved spermatocytes (Fig. 5 B–D, arrows). Remarkably, we observed that Speedy A–Ac (amino acids 1–199) could also rescue the telomere attachment defect in Spdya<sup>−/−</sup> spermatocytes and could colocalize with TRF1 (Fig. 5 E–G, arrows). Furthermore, Speedy A consisting of the Ringo domain plus 3 aa of the N terminus (amino acids 64–199) was also sufficient to localize to telomeres and rescue the attachment phenotype in Spdya<sup>−/−</sup> spermatocytes (Fig. 5 K–M, arrows). In contrast, the Speedy A fragment consisting of only the Ringo domain (amino acids 67–199) failed to localize to telomeres in Spdya<sup>−/−</sup> spermatocytes, and the internal telomere phenotype did not change upon electroporation of this fragment (Fig. 5 H–J, arrows). These results suggest that the amino acid 64–199 fragment of Speedy A contains the functional domain that facilitates telomere attachment to the NE. This domain is conserved among species and covers the distal portion of the N terminus and the Ringo domain (14, 16), and we call this domain the “telomere localization domain.”

**Localization of Cdk2 on Telomeres Depends on Its Interaction with Speedy A.** To determine if the binding of Cdk2 to Speedy A is of any importance for the localization of Cdk2 to the telomere–NE complex, we expressed GFP-tagged wild-type and mutated p39 Cdk2,
where its Lysine$^{33}$ (Lys$^{33}$), Arginine$^{50}$ (Arg$^{50}$), and Arginine$^{150}$ (Arg$^{150}$) residues were separately mutated to Alanine (Ala), by electroporation into wild-type mouse testes. A previous in vitro study has reported that the Lys$^{33}$, Arg$^{50}$, and Arg$^{150}$ residues of Cdk2 are required for Cdk2's binding to XRINGO, the Xenopus homolog of mouse Speedy A (18). Western blot results showed that these mutant Cdk2 forms can all be expressed in 293T cells (Fig. S8C), but they cannot bind or only weakly bind to Speedy A in 293T cells (Fig. S8D).

After electroporation into mouse testes, we found that p39 Cdk2 was localized to the telomeres (Fig. S8C–E, arrows) and on the XY body (Fig. S8C and E, arrowheads), as previously reported (9). In contrast, the p39 Cdk2 mutants Lys$^{33}$Ala, Arg$^{50}$Ala, and Arg$^{150}$Ala failed to localize to telomeres (Fig. S8F–N, arrows). These results suggest that Cdk2 binding to Speedy A is indispensable for Cdk2's localization to telomeres.

**Discussion**

Mammalian Speedy A, the most conserved member of the mammalian Speedy family, is highly expressed in mouse and human testes, but its expression has also been detected in various other human tissues and cell lines (14). For example, human Speedy A (Spy1) levels are tightly regulated during the proliferation of mammary progenitor cells, and are enriched in malignant human glioma cells, suggesting important roles for Speedy A in somatic cell cycle regulation in humans (31, 32). The Cdk-binding Ringo domain of Speedy A is 98% identical in mice and humans (14). In vitro studies on mouse Speedy A have shown that although the Ringo domain is essential for Cdk2 binding, activation of Cdk2 is through the C terminus of Speedy A (33).

In this study, we have found that in mice, Speedy A is specifically expressed in male and female germ cells at meiotic prophase I and is localized to the telomeres that have attached to the NE. Deletion of Speedy A in mice causes meiotic prophase I arrest, leading to male and female infertility. In Spdy$^{-/-}$ germ cells, telomeres could not efficiently attach to the NE in meiotic prophase I, and were thus arrested at a zygotene-like stage before the occurrence of characteristic pachytene events, such as telomeric cap exchange, homologous synopsis, and recombination.

A very recent study has also reported a similar phenotype in RingoA (i.e., Speedy A) knockout mice (19). However, in the present study we have further identified a TLD (amino acids 64–199) on Speedy A that covers the distal N terminus and the Ringo domain. It seems that this TLD of Speedy A is sufficient for mediating the initial formation of the telomere complex. Surprisingly, the TLD does not include the Cdk2-activation domain of Speedy A, implying that Cdk2 activation by Speedy A might not be necessary for mediating the initial telomere attachment to the NE in meiotic prophase I. On the other hand, the Speedy A fragment consisting of only the Cdk-binding Ringo domain without the distal N-terminal region was unable to localize to telomeres or rescue the phenotype in Spdy$^{-/-}$ spermatocytes. Therefore, we believe that the TLD of Speedy A plays a fundamental role in mediating the initial formation of the telomere–NE complex.

The involvement of the Cdk-binding Ringo domain indicates that not only Speedy A, but also Cdk2, are likely to function as structural components of the telomere complex. Indeed, Cdk2 proteins carrying mutations in key residues for binding to Speedy A are not localized on telomeres, indicating that binding to Speedy A is a prerequisite for Cdk2's telomere localization. It is possible that the primary role of Speedy A during telomere attachment is to act as a structural protein that anchors Cdk2 on telomeres. We propose that the interdependent binding between Speedy A and Cdk2 is of great importance for the initial telomere–NE complex assembly during early meiotic prophase I. It is likely that after Cdk2 is initially recruited onto telomeres by Speedy A, its kinase activity is further activated.

The kinase activity of Cdk2 on telomeres can be activated by Speedy A, but it is likely that other cyclins also activate the telomeric Cdk2, which in turn regulates telomere-led chromosome movements on the NE, possibly through phosphorylation of SUN1 (19, 34). It has been reported that E-type cyclins (E1 and E2) also form complexes with Cdk2 in mouse spermatocytes, and the knockout mice for Ccn1 and Ccn2 partially mimic the phenotypes seen in Cdk2$^{-/-}$ spermatocytes, suggesting the involvement of canonical cyclins in the activation of Cdk2 during meiosis (35, 36). In recent years, several meiosis-specific structural units that mediate chromosome attachment on the telomeric end have been identified in mice, including SUN1, KASH5, TERB1/2, and MAJIN (4–6, 8). Our present study suggests that Speedy A, which was previously known solely as a Cdk activator, has a structural function in the formation of the telomere complex during meiotic prophase I in mammals. The binding of Cdk2 to Speedy A might function as the initial structural component for the assembly of the telomere–NE complex. Our study thus extends the function of Speedy A and Cdk2 from just being cell cycle
kinases to now having roles as telomere component proteins during meiotic prophase I.

Materials and Methods

Detailed methods are described in SI Materials and Methods.

Animals

The bacterial artificial chromosome (BAC) DNA used to generate the Spdya targeting vector was obtained from a 129SV mouse BAC library (Invitrogen; Bac-RP23-456J14). Exon 2 of the Spdya gene was targeted and flanked by a loxP site at its 5’ end and a FRT-Neo-FRT-loxP cassette on its 3’ end using bacterial recombination, as previously reported (37). The Spdya targeting vector was linearized with PvuI and introduced into CJ7 ES cells by standard electroporation method. G418 and FIAU double-resistant ES cell clones were selected and analyzed using Southern blot hybridization of StuI/PacI-digested genomic DNA. Four independently targeted ES cell clones were injected into C57BL/6 blastocysts to produce the chimeras. Mice with the Spdya<sup>−/−</sup> allele of C57BL/6J background were crossed with Zp3-Cre transgenic mice of C57BL/6J background (23, 24) to delete exon 2 and generate Spdya<sup>−/−</sup> mice.

Mice were housed under controlled environmental conditions with free access to water and food. Illumination was on between 6:00 AM and 6:00 PM. Polyclonal antibodies were raised as described in Supporting Information.

Experimental protocols were approved by the regional ethical committee of the University of Gothenburg, Sweden, and by the Institutional Animal Care and Use Committee at the Biological Research Center animal facility at Biopolis, Singapore (for mice: #140927; for rabbits: #150998).

Preparation of Female Germ Cells by FACS

The embryonic gonads were harvested from 11.5-, 14.5-, 15.5-, 17.5-, 18.5-, and 19.5-dpc embryos. Gonads were cleaned of nongonadal tissues and digested for 30 min at 37 °C with 40 U/mL collagenase. After pipetting, the GFP<sup>+</sup> female germ cells were sorted by FACS in a BD Biosciences FACSRi cytometer. The isolated female germ cells were confirmed by their GFP fluorescence.

Exogenous Expression of Spdya cDNAs in Testes

Different fragments of Spdya were cloned into the pcAG-GFP vector (Addgene #11150). Plasmid DNA was injected into live mouse testes, and the exogenous expression was achieved by electroporation, as previously described (30). Spermatocyte cells expressing the exogenous genes were detected by immunostaining of GFP.

Acknowledgments

We thank Dr. Yoshinori Watanabe from the University of Tokyo, Japan, for providing the TERB1 and MAJIN antibodies. We also acknowledge the Centre for Cellular Imaging at University of Gothenburg. This study was supported by grants from the Jane and Dan Olssons Foundation, the LUA/ALF-medel Västra Götalandsregionen, AFA Insurance, the Swedish Research Council, and the Swedish Cancer Foundation, Sweden (all to K.L.). P.K. was supported by the Biomedical Research Council of A*STAR (Agency for Science, Technology, and Research), Singapore. J.-A.G. was supported by Robert A. Welch Foundation Grant E-0004.
Supporting Information

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SI Materials and Methods

Western Blot, Immunoprecipitation, and Histone H1 Kinase Assay. To prepare extracts, testes were collected from male C57BL/6 mice and suspended in RIPA buffer supplemented with Complete Protease Inhibitor (Roche) and Phosphatase Inhibitor (Roche). After homogenization, the cell extracts were centrifuged at 20,000 × g for 20 min at 4 °C. The supernatant extracts were used for immunoprecipitation and Western blots. Antibodies against Speedy A were raised by injecting subcutaneously 500 μg of purified mouse GST-Speedy A into two New Zealand White rabbits on days 0, 14, 35, 56, 77, and 98. Rabbits were bled on days 49, 70, 91, and 112 and serum was prepared. The IACUC protocol for raising polyclonal antibodies in rabbits is #150998. The rabbit anti-mouse Speedy A serum was used at 1:500. Rabbit anti-Cdk2 (1:1,000; #ab7954) was purchased from Abcam. Mouse anti-GFP (1:1,000; #sc-9996) was purchased from Santa Cruz. Mouse anti-β-actin (1:5,000; #A5316) and rat anti-HA (1:1,000; #11867423001) were purchased from Sigma-Aldrich.

For Cdk2 immunoprecipitation, 3 μg of anti-Cdk2 or control IgG was added and the extracts were incubated overnight at 4 °C, followed by incubation with Protein A Sepharose (GE Healthcare) for 1 h at 4 °C. The Western blots were repeated at least three times.

For transfection into 293T cells, different forms of Speedy A and Cdk2 cDNA constructs were transfected by Lipofectamine (Invitrogen, #11668019). The cells were harvested after 24 h of transfection. A total of 30-μg cell lysate was used for Western blot and 1mg cell lysate was used for immunoprecipitation.

Histone H1 kinase activity assays were performed as described previously (10). The radioactive signal was detected by an FLA 7000 phosphorimager (Fujifilm).

RT-PCR. Total RNA was extracted from about 10,000 female primordial germ cells by using the RNeasy Mini Kit (Qiagen). The first-strand cDNA was synthesized using the iScript TM cDNA synthesis kit (Bio-Rad). The PCR was performed with specific primers for Spdya (5′-TCTTTTGGCTATGACCTTTGTT-3′ and 5′-TTCTG-TCCGAGTTGGTC-3′). The housekeeping gene Gapdh (5′-GTCCGTAGACAAATGTTGA-3′ and 5′-TGATGTCCTGA-CAATCTTGAG-3′) was used to normalize the expression.

Histological Analysis. Tissues were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin-embedded tissues were cut into 5-mm sections and rehydrated. The sections were stained with hematoxylin for morphological analysis. Alternatively, for immunostaining, sections were subjected to antigen retrieval by heating in buffer (10 mM sodium citrate pH 6.0, 0.05% Tween 20).

Germ Cell Spreading and Immunostaining. Oocyte and spermatocyte spreading was performed using embryonic day 17.5 oocytes and PD18 testes, respectively. Spreads were prepared as previously described (22). Structurally preserved spermatocytes were prepared as described previously (38).

For immunostaining, standard protocols were used with the following primary antibodies: rabbit anti-DDX4 (1:200 dilution; Abcam #ab13840), mouse anti-SCP3 (1:500; Abcam #ab97672), rabbit anti-SCP1 (1:300; Abcam #ab15087), rabbit anti-SCP3 (1:500; Abcam #ab15093), mouse anti-MLH1 (1:50; BD Biosciences #554073), rabbit anti-γH2AX (1:200; Abcam #ab2893), mouse anti-Cdk2 (1:50, Santa Cruz #sc-6248), mouse anti-TRF1 (1:100; Abcam #ab10579); rabbit anti-TRF2 (1:200; Novus Biologicals #NB110-57130), and rabbit anti-SUN1 (1:200; Abcam #ab103021). Rabbit anti-Speedy A antibody (1:200) was made by the P.K. laboratory. Rat anti-SYCP3, rabbit anti-TERB1, and rabbit anti-MAJIN antibodies were kindly provided by Yoshinori Watanabe, Tokyo University, Japan. Primary antibodies were detected with Alexa Fluor 488-, 594-, or 647-conjugated goat secondary antibodies (1:100; Thermo Fisher Scientific #A11008, #A11007, #A21235).

Protein Purification. For coexpression of His-Cdk2 and Speedy A, cDNA encoding mouse Cdk2 (variant 1, for p39 Cdk2) and Spdya (amino acids 1–310 or 1–199) were cloned into pCOLADuet-1 (Novagen). The plasmids were transformed into BL21-Codon Plus (DE3) and Ni-NTA beads for HIS-tags (Qiagen) were used to purify the His-tagged proteins.

Imaging. Paraffin sections stained with hematoxylin were imaged with a Zeiss Axio Scope A1 upright microscope. Paraffin sections stained with immunofluorescent dyes were imaged with a Carl Zeiss Axio Observer Z1 inverted microscope. Imaging and analyses of spread germ cells were carried out with a Carl Zeiss LSM-700 Axios Observer Z1 inverted confocal microscope. For superresolution structured illumination microscopy (SR-SIM), the ELYRA PS.1 LSM780 setup from Zeiss (Carl Zeiss) was used. SR-SIM images were taken with the 63x/1.4 Plan-Apochromat oil-immersion objective. Z-stacks with an interval of 100 nm were used to scan the whole cell in SR-SIM. Because the SR-SIM images were acquired using different emission filter sets for the different color channels, multicolor beads were used to measure the possible misalignment between channels. The individual channels in all images were then aligned accordingly in the x-, y-, and z-directions. For acquisition, superresolution processing, channel alignment, and 3D reconstruction, the Zen 2011 software (Carl Zeiss) was used.
Fig. S1. Speedy A is localized to telomeres until the diplotene stage of prophase I. (A–I) Localization of Speedy A in PD25 wild-type spread spermatocytes. Telomeres were stained for TRF1. (A–C) In pachytene spermatocytes, Speedy A was colocalized with TRF1 on telomeres (arrows). Speedy A was also localized along the XY body as a linear element (arrowheads). (D–F) In diplotene spermatocytes, Speedy A was absent from telomeres (arrows). (G–I) Speedy A was completely absent from telomeres in metaphase spermatocytes (arrows). (J–O) Localization of Speedy A in 17.5-dpc wild-type spread oocytes. Telomeres were stained for TRF1. (J–L) In pachytene oocytes, Speedy A was colocalized with TRF1 on telomeres (arrows). (M–O) In diplotene oocytes, Speedy A was not detectable on telomeres (arrows).
Fig. S2. Generation of Spdyaflox/flox mice. (A) Exon 2 of the Spdyaflox gene was targeted and flanked by a loxP site at its 5′ end and a FRT-Neo-FRT-loxP cassette at its 3′ end. The Spdyaflox targeting vector was linearized with PvuI and introduced into CJ7 ES cells by a standard electroporation method. (B) Genomic DNA isolated from double-selected ES cell colonies was digested with StuI/PacI and analyzed by Southern blot hybridization. Wild-type DNA yields 15.2- and 14-kb bands after double digestion (arrows). Knockout mouse DNA yields a 9-kb band (arrowhead). (C) Genomic DNA isolated from double-selected ES cell colonies was digested with BamHI/PacI and analyzed by Southern blot hybridization. Wild-type DNA yields a 6-kb band. Floxed mouse DNA yields a 3-kb band (arrowhead).
Fig. S3. Normal oocyte maturation after deletion of Spdy. (A and B) As in Spdy<sup>flox/flox</sup> oocytes (A, arrow), normal germinal vesicle breakdown (GVBD) in Spdy<sup>flox/flox</sup>; Zp3-Cre oocytes (B, arrow) was observed upon release from the follicular environment. (C and D) Both Spdy<sup>flox/flox</sup> oocytes (C) and Spdy<sup>flox/flox</sup>; Zp3-Cre oocytes (D) had normal first polar body (PB1) extrusion (PBE) (arrows). A total of 50 oocytes from each group was analyzed, and representative images are shown. (E) Comparison of the cumulative number of pups per Spdy<sup>flox/flox</sup> female (black line) and per Spdy<sup>flox/flox</sup>; Zp3-Cre female (red line) (n = 4 for Spdy<sup>flox/flox</sup> mice, n = 6 for Spdy<sup>flox/flox</sup>; Zp3-Cre mice). The Spdy<sup>flox/flox</sup>; Zp3-Cre mice display normal fertility.
Fig. S4. Homologous synapsis, DNA DSB repair, homologous recombination, and telomere tethering to the LINC complex are impaired in Spdya−/− spermatocytes. (A) In Spdya+/+ pachytene spermatocytes, SYCP3 and SYCP1 overlapped on autosomal chromosomes (arrow). SYCP1 was absent from unsynapsed regions of the XY body (arrowhead). (B) In Spdya−/− zygotene-like spermatocytes, SYCP1 was partially formed (arrowhead), indicating impaired synapsis. (C) In Spdya+/+ pachytene spermatocytes, γH2AX was restricted to the XY body as an indication that DNA DSB repair had been completed on autosomal chromosomes (arrow). (D) In Spdya−/− zygotene-like spermatocytes, γH2AX was observed along all chromosome axes (arrowheads). (E) In Spdya+/+ pachytene spermatocytes, the chiasmata marker MLH1 was observed at sites of recombination (arrow). (F) In Spdya−/− zygotene-like spermatocytes, MLH1 was absent from chromosomes (arrowhead), indicating that homologous recombination did not occur in Spdya−/− spermatocytes. (G–L) In Spdya+/+ pachytene spermatocytes, TERB1, MAJIN, and SUN1 are localized to telomeres on the NE (G, I, and K, arrows). In Spdya−/− zygotene-like spermatocytes, TERB1 and MAJIN are localized to telomeres on the NE (H and J, arrows), but SUN1 is observed along the NE as a polarized cap (L, arrow).
Fig. S5. Speedy A maintained p39 Cdk2 expression in testes, and Speedy A might recruit Cdk2 to the NE during meiotic prophase I. (A) Western blot for Speedy A and Cdk2 in PD8 and PD17 testes from Spdyα+/+ and Spdyα−/− mice. At PD17, there was an increase of Speedy A and p39 Cdk2 in Spdyα+/+ testes, but no increase of p39 Cdk2 was seen in PD17 Spdyα−/− testes. β-Actin was used as the loading control, and 40-μg lysate was loaded in each lane. The experiments were repeated at least three times. (B) Cdk2 activity was decreased in Spdyα−/− testes, most likely because of the lower level of p39 Cdk2. Coomassie blue staining of H1 was performed as a loading control for the substrates. Immunoblotting (IB) of Cdk2 for the immunoprecipitation (IP) samples was used as a control for the decreased p39 Cdk2 level in Spdyα−/− testes. The experiments were repeated at least three times. (C) Speedy A can be pulled down by IP with anti-Cdk2 antibody, suggesting that Speedy A interacted with Cdk2 in male germ cells. A total of 2-mg testis lysate was used for the Cdk2 IP, and IgG was used as a negative control. The experiments were repeated three times. (D–F) In preleptotene cells, Speedy A (white arrows) was observed on the NE, whereas Cdk2 was absent (yellow arrows). (G–I) In leptotene cells, several Speedy A and Cdk2 signals overlapped on the NE (arrowheads). However, some telomeres displayed Speedy A signal but not Cdk2 signal (arrows). (J–L) In zygotene and pachytene cells, Speedy A and Cdk2 signals overlapped on the NE (arrowheads). However, Cdk2 was present at recombination sites (green arrows), whereas Speedy A was absent (white arrows). (P–R) In Spdyα−/− leptotene cells, Cdk2 was not observed on telomeres (arrowheads).
The C terminus of Speedy A is essential for Cdk2 activation. Cdk2 and Speedy A (full length) and Cdk2 and Speedy A–ΔC (Speedy A missing amino acids 200–310) were coexpressed in BL21 bacteria. The Speedy A–Cdk2 complex was purified via the His tag on Cdk2 and used in kinase assays with histone H1 as the substrate. Western blot results indicated that the amount of Cdk2-His was comparable in each complex, and both Speedy A and Speedy A–ΔC were successfully pulled down. The subsequent kinase assay demonstrated that H1 could be phosphorylated by His-purified Cdk2-Speedy A protein, but not by His-purified Cdk2-Speedy A–ΔC or Cdk2 alone, proving that the C terminus of Speedy A is indispensable for Cdk2 activation. Western blot of Cdk2 and Speedy A and Coomassie blue staining of H1 were used as controls. The experiments were repeated three times.
Fig. S7. Studies of the minimum Speedy A fragment that is required for its localization to telomeres. The different regions of Speedy A cDNA were cloned into the pCAG-GFP vector and expressed by electroporation in wild-type testes. (A) Western blot showing that different fractions of Speedy A protein were correctly expressed. 293T cells were transfected with plasmids containing different regions of Speedy A cDNA, and the cells were harvested 24 h after transfection. A total of 30μg cell lysate was used for Western blot, and β-actin was used as the loading control. The correct bands are marked by asterisks (*). (B–D) Immunofluorescence of GFP, TRF1, and SYCP3 indicate that full-length Speedy A protein can localize to telomeres after in vivo expression in wild-type testes (arrows). (E–G) Speedy A–ΔC GFP protein showed telomeric localization as indicated by TRF1 and SYCP3 costaining (arrows). (H–J) Speedy A–Ringo-only protein did not localize to telomeres (arrows). (K–M) Speedy A–TLD protein was able to localize to telomeres as indicated by TRF1 and SYCP3 costaining (arrows).
Fig. S8. Localization of Cdk2 on telomeres is mediated by its interaction with Speedy A. (A) p39 Cdk2 cDNAs carrying point mutations were cloned into the pCAG-GFP vector and expressed in 293T cells, a total of 30-μg cell lysate was used for Western blot and β-actin was used as the loading control. (B) HA-Speedy A and p39 Cdk2-GFP plasmid or p39 Cdk2-GFP cDNAs carrying point mutations were cotransfected into 293T cells and IP using anti-HA antibody was performed. Western blot indicated that only wild-type p39 Cdk2 is able to interact with Speedy A, and the p39 Cdk2 mutants Lys\textsuperscript{33}Ala, Arg\textsuperscript{50}Ala, and Arg\textsuperscript{150}Ala showed no or very weak interaction with Speedy A in 293T cells. A total of 1-mg cell lysate was used for IP. (C–E) p39 Cdk2 cDNA in pCAG-GFP vector was expressed by electroporation into wild-type testes. Immunofluorescence of GFP, TRF1, and SYCP3 illustrating that p39 Cdk2 protein could localize to telomeres after in vivo expression in wild-type testes (arrows). Localization is also observed on the XY body (arrowheads). (F–H) Replacement of Lys\textsuperscript{33} with Ala in the p39 Cdk2 sequence prevents the protein from localizing to telomeres (arrows). (I–K) Replacement of Arg\textsuperscript{50} with Ala in the p39 Cdk2 sequence diminishes p39 Cdk2’s localization to telomeres (arrows). (L–N) Replacement of Arg\textsuperscript{150} with Ala also prevents p39 Cdk2 from localizing to telomeres (arrows).