Oligo-astheno-teratozoospermia in mice lacking *Cnot7*, a regulator of retinoid X receptor beta

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Spermatogenesis is a complex process that involves cooperation of germ cells and testicular somatic cells. Various genetic disorders lead to impaired spermatogenesis, defective sperm function and male infertility¹. Here we show that *Cnot7*^{-/-} males are sterile owing to oligo-astheno-teratozoospermia, suggesting that *Cnot7*, a CCR4-associated transcriptional cofactor², is essential for spermatogenesis. Maturation of spermatids is unsynchronized and impaired in seminiferous tubules of *Cnot7*^{-/-} mice. Transplantation of spermatogonial stem cells from male *Cnot7*^{-/-} mice to seminiferous tubules of *Kit* mutant mice (*Kit*^{W/W-v}) restores spermatogenesis, suggesting that the function of testicular somatic cells is damaged in the *Cnot7*^{-/-} condition. The testicular phenotypes of *Cnot7*^{-/-} mice are similar to those of mice deficient in retinoid X receptor beta (*Rxb*)³. We further show that *Cnot7* binds the AF-1 domain of *Rxb* and that *Rxb* malfunctions in the absence of *Cnot7*. Therefore, *Cnot7* seems to function as a coregulator of *Rxb* in testicular somatic cells and is thus involved in spermatogenesis.

Genetic analyses in yeast suggest that CAF1, a component of the CCR4-NOT complex, has multiple roles in regulating transcription⁴. In addition, proteins in the CCR4-NOT complex are involved in mRNA metabolism in yeast^{5,6}. Two mammalian homologs of yeast CAF1 are known: *Cnot7* (also called CAF1) and *Cnot8* (also called CALIF)^{7,8}. Both are expressed in a variety of tissues, with relatively high expression of *Cnot7* in lung, liver and thyroid gland^{7,8}. *Cnot7* interacts with members of the TOB-BTG antiproliferative family, which comprises Tob1, Tob2, Btg1, Btg2 (also called PC3 and TIS21), Btg3 (also called ANA) and Btg4 (also called PC3B; refs. 7,9,10). The proteins of this family are implicated in regulation of transcription^{11–14}.

To elucidate the physiological role of mammalian *Cnot7*, we produced mutant mice lacking the gene *Cnot7* by means of homologous recombination (Fig. 1a–d). Homozygous *Cnot7* knockout (*Cnot7*^{-/-})

mice were alive at birth and born at the predicted mendelian frequency. Adult *Cnot7*^{-/-} mice had normal health, size and behavior, except that male *Cnot7*^{-/-} mice were sterile. *Cnot7*^{+/-} males had normal fertility and *Cnot7*^{-/-} females produced average-size litters (6.3 ± 2.1 offspring per litter; n = 14 litters).

There were no gross anatomical differences in the seminal vesicles and prostates among *Cnot7*^{+/+}, *Cnot7*^{+/-} and *Cnot7*^{-/-} males, but the testes of *Cnot7*^{-/-} mice were smaller than those of *Cnot7*^{+/+} or *Cnot7*^{+/-} mice (Fig. 1e). *Cnot7*^{-/-} mice produced only 7% as much sperm as *Cnot7*^{+/+} or *Cnot7*^{+/-} mice (Fig. 1f), and their spermatozoa beat less vigorously and generated less forward momentum (Fig. 1g). Almost all spermatozoa from *Cnot7*^{-/-} mice had irregularly shaped heads, abnormally arranged mitochondria and erroneously attached flagella (Fig. 1h,i). Electron microscopic analysis detected ultrastructural components, such as condensed chromatin, acrosomes and flagella, including axoneme, mitochondrial sheath, outer dense fibers and fibrous sheath, in spermatozoa from *Cnot7*^{-/-} mice. But their arrangements were irregular and maturation of sperm was abnormal (Fig. 1i). The degree of morphological irregularity varied: spermatozoa of *Cnot7*^{-/-} mice were round-headed (73%), tapered-headed (20%), symplast (5%) or nearly normal (2%). We also observed malformation of spermatids in the seminiferous tubules (see Fig. 3d). Taken together, these data indicate that oligo-astheno-teratozoospermia (low sperm number and motility and abnormal sperm morphology) underlies the sterility of *Cnot7*^{-/-} males.

To further analyze sperm competence for fertilization, we carried out *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) experiments. For the IVF experiment, we freed oocytes from cumulus cells and the zona pellucida, because epididymal spermatozoa of *Cnot7*^{-/-} mice had poor motility and were unable to penetrate these egg layers. Even under these experimental conditions, spermatozoa of *Cnot7*^{-/-} mice hardly fertilized oocytes, whereas *Cnot7*^{+/-} spermatozoa had normal fertilizing ability (Table 1). Direct injection of

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Figure 1 Targeted disruption of *Cnot7* and defects in maturation and morphology of sperm in *Cnot7*^{-/-} mice. **(a)** Schematic diagram of the targeting vector and the wild-type and targeted alleles. Black boxes show exons 2, 3 and 4 of *Cnot7*. *nLacZ*, *lacZ* coding sequence with nuclear localization signal; *neo*, neomycin resistance gene driven by the thymidine kinase promoter; *DT-A*, diphtheria toxin A-chain gene; RV, *EcoRV*; Nc, *NcoI*; K, *KpnI*; Xh, *XhoI*. **(b)** The 5' external probe was used for Southern-blot analysis to detect homologous recombination. Tail DNAs from F1 progeny of heterozygote intercrosses were digested with *NcoI* and subjected to Southern-blot hybridization. **(c)** Northern-blot analysis of total RNAs (10 µg) from testes of *Cnot7*^{+/+}, *Cnot7*^{+/-} and *Cnot7*^{-/-} mice with the 701-bp fragment (nucleotides 158–858) of the human *CNOT7* cDNA. **(d)** Western-blot analysis of protein extracts from *Cnot7*^{+/+}, *Cnot7*^{+/-} and *Cnot7*^{-/-} MEFs with antibodies to *Cnot7*. **(e)** Comparison of testis weight in three 5-month-old *Cnot7*^{+/-} and *Cnot7*^{-/-} mice. The organs were trimmed free of fat and weighed. **(f,g)** Number and motility of sperm prepared from three 5-month-old *Cnot7*^{+/-} and *Cnot7*^{-/-} mice. Sperm counts **(f)** and percentages of motile sperm **(g)** were determined visually by microscopy. Error bars represent s.d. Statistical significance (**P* < 0.01) in each assay was assessed by Student's *t*-test. **(h)** Morphology of spermatozoa. Spermatozoa were collected from the cauda epididymides of *Cnot7*^{-/-} mice and photographed without fixation under Nomarski optics (magnification, ×600). **(i)** Transmission electron micrographs of spermatozoa from the cauda epididymides in male *Cnot7*^{+/+} (top) and *Cnot7*^{-/-} (middle and bottom) mice. In *Cnot7*^{+/+} mice, head was normally hooked in shape and nucleus (Nu) was condensed. Acrosome (Ac) and mitochondria (Mt) were also normally formed. In *Cnot7*^{-/-} mice, nuclei of spermatozoa were condensed but did not form the characteristic hooked shape. Acrosomes were expanded and attached to nucleus. Abnormal arrangement and localization of mitochondria were apparent and flagella (F) were incorrectly attached. The middle panel shows localization of condensed nucleus in the cytoplasmic compartments. The differences in body weight, sperm count, motility and morphology between *Cnot7*^{+/+} and *Cnot7*^{+/-} mice were not significant. Scale bars, 1 µm.

spermatids from *Cnot7*^{-/-} mice into oocytes by conventional ICSI resulted in normal fertilization and production of pups after embryo transfer. The efficiencies were comparable to those after ICSI using control spermatids (Table 1), indicating that spermatids from *Cnot7*^{-/-} mice had an adequate set of haploid genome and full oocyte-activating capacity. We conclude from these data that *Cnot7*^{-/-} male germ cells have defects in postmeiotic modifications that are essential for enabling natural delivery of the paternal genome into the oocytes.

Detailed histological analysis of seminiferous tubules from *Cnot7*^{-/-} mice showed a reduction in the number of late spermatids and the presence of large, round, clear vacuoles (Fig. 2a–g). In severe cases,

most germ cells were lost in seminiferous tubules of *Cnot7*^{-/-} mice (Fig. 2f). We often observed multiple generations of elongated spermatids in the same section of seminiferous tubules of *Cnot7*^{-/-} mice (Fig. 2h). The data indicate that maturation of spermatids in *Cnot7*^{-/-} mice is unsynchronized. Spermatogenesis in *Cnot7*^{-/-} mice was also distinguished from that in control mice by the presence of syncytial (multinucleated) germ cells (Fig. 2h) and a greater number of apoptotic cells (Fig. 2i,j). Lipids were unusually accumulated in the cytoplasm of Sertoli cells of adult *Cnot7*^{-/-} mice as compared with control mice (Fig. 2c,g,k,l), suggesting that deficiency of *Cnot7* induces a metabolic defect in Sertoli cells.

Table 1 *In vitro* and *in vivo* assessment of fertilizing ability of spermatozoa

Method	<i>Cnot7</i> genotype	Number of oocytes used	Number of oocytes fertilized (%)	Number of embryos transferred	Number of embryos implanted (%)	Number of offspring (%)
IVF	-/-	35	1 (3) ^a	—	—	—
	+/-	23	15 (65) ^a	—	—	—
ICSI	-/-	133	122 (91.2) ^b	110	72 (65.5) ^b	33 (30.0) ^b
	+/-	146	124 (84.9) ^b	124	89 (71.8) ^b	53 (42.7) ^b

^a*P* < 0.01. ^b*P* > 0.05 (χ^2 test).

Figure 2 Unusual histology of seminiferous tubules of *Cnot7*^{-/-} mice. (a,b,e,f) Histological sections of seminiferous tubules prepared from 5-month-old *Cnot7*^{+/-} (a,b) and *Cnot7*^{-/-} (e,f) males were stained with hematoxylin and eosin. Asterisks indicate typical vacuoles. Scale bars: a,e, 100 μ m; b,f, 50 μ m. (c,d,g,h) Impairment of sperm maturation in seminiferous tubules of *Cnot7*^{-/-} mice. Histological sections of seminiferous tubules prepared from 2-month-old *Cnot7*^{+/-} (c,d) and *Cnot7*^{-/-} (g,h) males were stained with toluidine blue. Asterisks, arrow and filled arrowheads indicate vacuoles, a syncytial cell and unusual accumulations of lipids in the cytoplasm of Sertoli cells, respectively. Spermatogenesis was well-synchronized in *Cnot7*^{+/-} males at stage VIII (c) and at stage XII (d) but was not well-synchronized in *Cnot7*^{-/-} males at stage VIII (g) and at stage XII (h), which featured multiple generations of spermatogenic cells (open arrowheads). Double arrowheads in d and h indicate secondary spermatocytes. Scale bars, 25 μ m. (i,j) More germ cell apoptosis in testes of *Cnot7*^{-/-} mice. Apoptotic cells in seminiferous tubules from 5-month-old *Cnot7*^{+/-} (i) and *Cnot7*^{-/-} (j) males were stained (brown) intensively by TUNEL. Arrows indicate apoptotic cells. Scale bars, 50 μ m. (k,l) Unusual accumulation of lipids (arrowheads) in the cytoplasm of Sertoli cells. Light micrograph showing spermatogenesis in testis from a *Cnot7*^{-/-} mouse was stained with toluidine blue (k). Transmission electron micrograph also shows a lipid droplet in Sertoli cells of testis from a *Cnot7*^{-/-} mouse (l). Scale bars: k, 10 μ m; l, 2 μ m.

β -galactosidase staining of seminiferous tubules from *Cnot7*^{+/-} mice showed that *Cnot7* was strongly expressed in Sertoli and Leydig cells (Fig. 3a) but only weakly expressed in early round spermatids, spermatocytes and spermatogonia. Because intimate interactions between germ cells and somatic support cells are essential for spermatogenesis, the impaired spermatogenesis in *Cnot7*^{-/-} mice may be caused by defects intrinsic to the support cells. To examine this possibility, we carried out spermatogonial stem cell transplantation^{15,16}. We transplanted germ cells from 8- to 10-week-old *Cnot7*^{-/-} males into the seminiferous tubules of WBB6F₁ W/W^v (*Kit*^{W/W-v}) mice, busulfan-treated nude (BALB/c) mice or irradiated C57BL/6 mice. The testes of these mice are hospitable for donor cell colonization because they lack endogenous germ cells and have functionally normal Sertoli cells¹⁵. By tracing expression of the *lacZ* transgene, we found that the transplanted *Cnot7*^{-/-} spermatogonial stem cells differentiated into spermatids in the seminiferous tubules of recipient mice after transplantation

(Fig. 3b,c). Morphologically normal spermatids (Fig. 3d) were aligned at the luminal side of seminiferous tubules in the *Kit*^{W/W-v} recipient testes by 3 months after transplantation (Fig. 3c,e). These results suggest that *Cnot7* functions in testicular somatic cells to establish the proper testicular microenvironment for spermatogenesis. Results of reciprocal transplantation experiments, in which germ cells from transgenic mice carrying the pCXN-eGFP transgene¹⁷ were transmitted into the seminiferous tubules of busulfan-treated *Cnot7*^{-/-} mice, were consistent with that conclusion (data not shown).

Several nuclear receptors including estrogen receptor α , androgen receptor, retinoic acid receptor α (Rara) and Rxrb are essential for spermatogenesis^{3,18-20}. Of these, Rxrb is expressed in Sertoli and Leydig cells^{3,21}. *Rxrb*^{-/-} male mice are sterile, owing to abnormal germ cell maturation that leads to oligo-astheno-teratozoospermia. In addition, Sertoli cells of *Rxrb*^{-/-} mice undergo a progressive accumulation of lipids³.

Figure 3 Macroscopic and histological appearances of seminiferous tubules of recipient mouse testes after transplantation of *Cnot7*^{-/-} germ cells. (a) Expression of *Cnot7* in testicular cells. β -galactosidase staining of testes from *Cnot7*^{+/-} mice indicates strong *Cnot7* expression in somatic cells. Arrows and arrowheads indicate Leydig and Sertoli cells, respectively. Scale bar, 50 μ m. (b) A testis from a transplanted recipient mouse showing β -galactosidase expression. β -galactosidase-positive (blue) stretches of seminiferous tubules are colonies from donor spermatogonial stem cells. (c) Histological section of *Kit*^{W/W-v} recipient seminiferous tubules stained with periodic acid-Schiff and hematoxylin. Arrow and arrowhead indicate colonized and noncolonized seminiferous tubules, respectively, after transplantation. Scale bar, 50 μ m. (d) Transmission electron micrographs showing a malformed head of elongating spermatid in testis of a *Cnot7*^{-/-} mouse (upper panel) and a normally formed head of elongating spermatid obtained from the transplantation experiment (lower panel). Nu, condensed nuclei; Ac, acrosome. Scale bars, 1 μ m. (e) Histological section of *Kit*^{W/W-v} recipient seminiferous tubules stained with toluidine blue. Scale bar, 10 μ m.

We expressed FLAG-tagged human CNOT7 in COS7 cells together with Rxrb or other nuclear receptors (Rxra, Rxrg, Rara and vitamin D receptor) fused to glutathione S-transferase (GST). GST pull-down assays with the cell lysates showed that CNOT7 interacted only with GST-Rxrb (Fig. 4a). This interaction was confirmed by immunoprecipitation experiments with lysates of TTE3 Sertoli cells²² (Fig. 4b). Rxra, Rxrb and Rxrg share a conserved DNA-binding domain (~95% homology) and C-terminal ligand-binding domain (AF-2, ~87% homology). In contrast, the sequence of the N-terminal domain (AF-1), which is involved in autonomous ligand-independent transcriptional activation, is divergent across family members²³. GST pull-down assays showed that the AF-1 domain of Rxrb interacted with CNOT7 whereas the DNA-binding and AF-2 domains did not (Fig. 4c).

We then examined whether Rxrb functions are affected in the absence of Cnot7. To measure the Rxrb-mediated transcription in the *Cnot7*^{-/-} condition, we used primary mouse embryonic fibroblasts (MEFs). We transfected an Rxrb expression vector and a luciferase reporter plasmid containing an RXRE-response element (RXRE)-coupled thymidine kinase promoter into wild-type or *Cnot7*^{-/-} MEFs. Luciferase reporter assays showed that Rxrb-mediated transcription in response to 9-*cis* retinoic acid was much lower in the absence of Cnot7 (Fig. 4d). In addition, electrophoretic mobility shift assays (EMSAs) showed that proteins from testes of *Cnot7*^{-/-} mice bound to the RXRE less efficiently than those from testes of *Cnot7*^{+/+} mice (Fig. 4e). Reintroduction of recombinant CNOT7 in the nuclear extracts of

testes of *Cnot7*^{-/-} mice resulted in substantial recovery of the ability to bind the RXRE (Fig. 4f). Taken together, these findings suggest that the AF-1 domain is responsible for the Rxrb-Cnot7 interaction and that Cnot7 contributes to Rxrb function.

In this study, we found *in vivo* evidence that abnormalities during spermatogenesis in testes of *Cnot7*^{-/-} mice are possibly caused by defects in the testicular somatic cells rather than in the germ cells. The function of testicular somatic cells is directed by pituitary gonadotropins secreted from the hypothalamic-pituitary axis. Among the gonadotropins, luteinizing hormone mainly stimulates testosterone production in the Leydig cells. Because serum testosterone level did not differ between male *Cnot7*^{+/+} and *Cnot7*^{-/-} mice (data not shown), hormonal regulation mediated through the hypothalamic-pituitary axis and Leydig cells seems to be little affected by the absence of Cnot7. The testicular phenotypes in *Cnot7*^{-/-} mice seem to be caused by the functional defect of Sertoli cells.

Although the RXR family members Rxra, Rxrb and Rxrg share structural similarities, genetic analyses suggest that each has distinct roles in mice²⁴⁻²⁶. Our data show that testicular phenotypes of male *Cnot7*^{-/-} mice are similar to those of *Rxrb*^{-/-} mice (vacuole formation, failure in alignment of late spermatids, multiple generations of spermatids, lipid accumulation in the cytoplasm of Sertoli cells and no apparent phenotypic abnormalities in other adult tissues), suggesting that Cnot7 functionally interacts with Rxrb but not with other family members. Indeed, our data show that Rxrb interacts physically and functionally

Figure 4 Physical and functional interactions between Cnot7 and Rxrb. **(a,c)** FLAG-tagged CNOT7 was coexpressed in COS7 cells with GST-fused nuclear receptors (NRs; mouse Rxra, mouse Rxrb, mouse Rxrg, mouse Rara and rat vitamin D receptor (Vdr); **a**) or GST-fused distinct portions of Rxrb (**c**). Total protein extracts were subjected to GST pull-down assay followed by immunoblotting (IB) with monoclonal antibody against FLAG (top panel). The lower two panels show expression of each indicated protein. **(b)** Proteins in the lysates of TTE3 Sertoli cells were immunoprecipitated with antibodies to Cnot7 (α Cnot7) or nonspecific IgG. The immunoprecipitates were subjected to immunoblotting (IB) with antibodies to Rxrb (upper panel) or to Cnot7 (lower panel). Filled arrowheads to the left indicate bands that interacted specifically with antibodies; open arrowheads to the right indicate bands with non-specific antibody binding. **(d)** Impairment of 9-*cis* retinoic acid (RA)-induced Rxrb-mediated transcription due to the absence of Cnot7. Wild-type and *Cnot7*^{-/-} MEFs were transfected with Rxrb expression plasmid together with RXRE-*luc* reporter plasmid. Cells were incubated in the absence or presence of 9-*cis* retinoic acid (1 M). Error bars represent s.d. Asterisk indicates a statistically significant difference between wild-type and *Cnot7*^{-/-} MEFs ($P < 0.01$). Statistical differences between groups were assessed by Student's *t*-test. **(e)** Effect of Cnot7 on Rxrb DNA-binding. Increasing amounts of nuclear extracts (1 g, +; 3 g, ++; 9 g, +++) isolated from testes of wild-type (lanes 2–4) or *Cnot7*^{-/-} mice (lanes 5–7) were incubated with a fixed amount of radioactively labeled response element for EMSA. The DNA-binding activity of Rxrb from testes of wild-type mice (lane 8) was compared with that of testes from *Cnot7*^{-/-} mice (lane 9) by supershift experiments with antibody to Rxrb. Levels of Rxrb expression were similar in testes from wild-type and *Cnot7*^{-/-} mice (data not shown). The quantity of nuclear extracts used was confirmed with the oligonucleotide probe containing κ B site from the mouse κ -light chain enhancer (lanes 10 and 11). **(f)** Functional associations between Cnot7 and Rxrb. Recombinant CNOT7 was produced in *E. coli* and added to the nuclear extracts of testes from *Cnot7*^{-/-} mice before EMSA (lane 4, right panel). The left panel shows Coomassie brilliant blue staining of the recombinant CNOT7 protein.

with CNOT7 through its AF-1 domain. Therefore, Cnot7 may function as a specific coregulator of Rxrb to contribute to spermatogenesis.

Little is known about the molecular mechanism of human infertility. It may be important to screen men with oligo-astheno-teratozoospermia for polymorphisms or mutations in *CNOT7* to assess whether *CNOT7* has a role in human infertility.

METHODS

Generation of *Cnot7*^{-/-} mice. We subcloned the 18-kb genomic DNA fragment of *Cnot7* into pBluescript. We replaced an exon of *Cnot7* that contains the first methionine with *lacZ* and a *pMC1neo poly(A)* fragment flanked by *loxP* sites to be inserted in-frame with the first methionine to generate the targeting vector. The DT-A fragment was ligated at the 3' end of the targeting vector for negative selection. We electroporated J1 embryonic stem cells with linearized targeting vector and subjected them to neomycin selection. We identified *Cnot7* targeted clones by Southern-blot hybridization with the probe shown in **Figure 1a**, injected these cells into C57BL/6J blastocysts and mated chimeric offspring with C57BL/6J mice. We intercrossed heterozygous F1 mice to produce homozygous *Cnot7*^{-/-} mice. We carried out all experiments with animals following guidelines for animal use issued by the Committee of Animal Experiments, Institute of Medical Science, University of Tokyo.

Conventional transmission electron microscopy. We fixed adult testes with 2% glutaraldehyde in 0.2 M cacodylate buffer. After washing them in the same buffer, we cut the tissues into small pieces, immersed them in the same fixative for 2 h at 4 °C, rinsed them and fixed them with OsO₄. The samples were dehydrated through graded ethanol and then embedded in Epon 812. We cut ultrathin sections on an ultramicrotome (model Ultracut E, Reihert-Jung) and stained them with uranyl acetate and lead citrate. We observed them with a JEOL 1200 EX (JEOL) transmission electron microscope.

IVF and ICSI. We collected mature oocytes from the oviducts of female B6D2F1 mice that had been superovulated by the injection of 5 IU of equine chorionic gonadotrophin followed 48 h later by 5 IU of human chorionic gonadotropin. We carried out zona-free IVF using epididymal spermatozoa as described²⁷. We fixed, stained and examined oocytes by phase-contrast microscopy 2 h after insemination. Oocytes with decondensed sperm nuclei were considered to be fertilized. For ICSI, we collected spermatogenic cells from the seminiferous tubules by a mechanical method and directly injected elongated spermatids at steps 9–11 into oocytes using a Piezo-driven micromanipulator²⁸. Oocytes that survived injection were cultured in potassium simplex optimized medium and those developing to the 2-cell stage were transferred into the oviducts of pseudopregnant ICR females. We obtained live offspring at term (day 19.5) by Caesarian section or after natural delivery.

Testes histomorphometry. We fixed testes in Bouin or 10% formalin neutral buffer solution, embedded them in paraffin and cut 7-mm sections. We dewaxed and stained sections with hematoxylin and eosin, with toluidine blue or by PAS reaction with standard procedures.

Analysis of apoptotic cells. We detected apoptotic cells in sections of mouse testes *in situ* by TUNEL assay with an ApopTag peroxidase kit (Intergen). We counterstained sections with diluted hematoxylin.

Production of recipient mice and spermatogonial stem cell transplantation. We obtained male WBB6F₁-W/W^m mice from Japan SLC. We injected 6-week-old BALB/cA Jcl-*nu* males (CLEA Japan) intraperitoneally with freshly prepared busulfan (44 mg per kg body weight). Busulfan-treated mice were devoid of endogenous spermatogenesis at the time of transplantation (~6 weeks after busulfan treatment). We irradiated (12 Gy) the lower halves of the bodies of 14- to 18-d-old C57BL/6N mouse pups (CLEA Japan) to eliminate endogenous germ cells in the testes and used these mice for transplantation experiments 2 d later²⁹. We prepared donor cell suspension (2–3 × 10⁷ cells ml⁻¹) from 8- to 10-week-old *Cnot7*^{-/-} mice by a two-step enzymatic digestion technique¹⁶ and introduced ~10⁶ into the seminiferous tubules of each recipient mouse. We identified donor-derived areas of spermatogenesis in testes of recipients by staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal).

Cell culture. We obtained MEFs from 14.5-d-old embryos by an established procedure¹⁴. We maintained MEFs, COS7 and TTE3 cells in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics.

Plasmids. We amplified cDNA fragments for various portions of GST-fused Rxrb by PCR. The AF-1 domain of Rxrb was described previously³⁰. Primer sequences for Rxrb mutants are available on request.

GST pull-down. For GST pull-down assays, we lysed cells with RIPA buffer as described⁹ 36 h after transfection. We purified GST fusion proteins with glutathione-Sepharose beads, resolved the bound proteins by SDS-PAGE and analyzed them by immunoblotting. Antibodies used for blotting were monoclonal antibody to FLAG (Sigma) and monoclonal antibody to GST (Santa Cruz Biotechnology).

Immunoprecipitation and immunoblotting. We solubilized TTE3 cells in Triton X-100 lysis buffer (0.5% Triton X-100, 50 mM Tris (pH 7.4), 10% glycerol, 100 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 1 M 9-*cis* retinoic acid and 25 M MG132) supplemented with protease inhibitor cocktails (Sigma). We incubated precleared lysates sequentially with polyclonal antibodies to Cnot7 and protein A-Sepharose (Amersham Biosciences). We washed the immunoprecipitates at least four times with lysis buffer. We raised rabbit polyclonal antibodies against Cnot7 using a keyhole limpet hemocyanin-conjugated synthetic peptide with the sequence SYVQNGTGNAYEEEEANKQS as immunogen and affinity-purified it. We used a monoclonal antibody to Rxrb for blotting, which was a gift from Perseus Proteomics.

Transactivation assay. For Rxrb-mediated transactivation assay, we cotransfected MEFs (5 × 10⁴ cells per well in 12-well tissue culture plates) with Lipofectamine (Invitrogen) and the following plasmids: (i) pSG5-Rxrb (0.2 g), (ii) pGL3-RXRE-Luc (reporter plasmid; 0.05 g) and (iii) pRL-TK (0.025 g). We analyzed cell extracts for luciferase activity with a Dual-Luciferase Reporter System (Promega). Transfection efficiency was standardized with an internal control plasmid, pRL-TK. Data are shown as the average ± s.d. of three independent experiments, each done in triplicate.

Preparation of nuclear extracts and EMSA. We isolated testes from 10- to 16-week-old *Cnot7*^{+/+} or *Cnot7*^{-/-} mice, washed them in ice-cold phosphate-buffered saline and suspended them in hypotonic buffer (10 mM HEPES buffer (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). We broke cells with ten strokes with a type A Dounce homogenizer and two gentle strokes with a type B Dounce homogenizer. We collected nuclei by centrifugation for 10 min at 600g and 4 °C and resuspended them in 500 μl of hypotonic buffer and added 250 μl of low salt buffer (20 mM HEPES buffer (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF). We then added 250 μl of high salt buffer (20 mM HEPES buffer (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF) dropwise. We extracted nuclei for 30 min at 4 °C with constant rotation, centrifuged the suspension for 30 min at 150,000g and 4 °C, collected the supernatant and used it for EMSAs. We used an RXRE composed of two half sites oriented as direct repeats with a 1-kb spacer element as the oligonucleotide probe. We incubated the nuclear extracts at room temperature for 20–30 min with the radioactively labeled oligonucleotide probe (40,000–60,000 c.p.m.) in 15 μl of binding buffer (15 mM Tris-HCl (pH 7.5), 75 mM NaCl, 1.5 mM EDTA, 15 mM DTT, 7.5% glycerol, 1 g l⁻¹ bovine serum albumin and 0.3% Nonidet P-40). We then resolved the resulting DNA-protein complexes by nondenaturing gel electrophoresis and visualized them by autoradiography. For supershift experiments, we used antibody to Rxrb (Santa Cruz Biotechnology; sc-831).

Protein cloning, expression, and purification. We cloned a DNA fragment encoding the full-length human CNOT7 protein into pGEX-6P-3 (Amersham Biosciences). We expressed the protein in *Escherichia coli* BL21, purified it by affinity chromatography on glutathione Sepharose 4B and cleaved it from GST with PreScission protease (Amersham Biosciences). The protein samples from vector-transfected *E. coli* were similarly treated (Vehicle).

Sperm count and motility analysis. We placed cauda epididymides in 0.1 ml of motile buffer (120 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄ and 1.3 mM CaCl₂). We minced the tissue with scissors and incubated it at 37 °C for 5 min to allow sperm dispersal.

ACKNOWLEDGMENTS

We thank J. Yanagisawa, Y. Sugitani, T. Nakazawa, M. Ohsugi, T. Tezuka, K. Semba and M. Noda for discussions; S. Kato for providing expression vectors for nuclear receptors and reporter plasmids containing response elements for nuclear receptors; N. Yanai for providing TTE3 cells; and H. Yamanaka, N. Kusaka, A. Nakamura, M. Yoneda, A. Moriya and F. Suzuki-Toyota for technical support. This work was supported by a Grant for Advanced Cancer Research from the Ministry of Education, Science, Sports, and Culture of Japan and grants from the Organization for Pharmaceutical Safety and Research of Japan, and from the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 21 January; accepted 17 March 2004

Published online at <http://www.nature.com/naturegenetics/>

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