NUP62: the target of an anti-sperm auto-monoclonal antibody during testicular development[1]

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Short title: Target of Ts4 during testicular development

Summary sentence: Ts4, a mouse anti-sperm auto-monoclonal antibody, reacts with a specific sugar moiety of glycoproteins containing bisecting GlcNAc. During testicular development after the birth, NUP62 is the main molecule possessing Ts4-reactive bisecting GlcNAc.

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Abstract

Ts4, an autosperm-monoclonal antibody (mAb) reacts with a specific oligosaccharide (OS) of glycoproteins containing bisecting *N*-acetylglucosamine residues. Ts4 reactivity was observed against epididymal spermatozoa, testicular germ cells, and the early embryo, but not against major organs in adult mice. In mature testis, Ts4 exhibits immunoreactivity with a germ cell-specific glycoprotein, TEX101, whereas the mAb immunoreacts with alpha-*N*-acetylglucosaminidase in the acrosomal region of cauda epididymal spermatozoa. Thus, Ts4 seems to react against different molecules throughout spermiogenesis via binding to its OS epitope. Since the Ts4-epitope OS is observed only in reproduction-related regions, the Ts4-reactive OS may play a role in the reproductive process. The aim of this study is to investigate the characteristics of the Ts4-reactive molecule(s) during testicular development. Ts4 reactivity was observed in testes from the prenatal period; however, its distribution changed according to the stage of maturation and was identical to that of the adult testes after 29-day-postpartum (dpp). Ts4 immunoreactivity was detected against a protein with 63-kDa in testis from 1- to 29-dpp. In contrast, Ts4 showed reactivity against some other glycoproteins after 29-dpp, including TEX101 at the 5-wk-old stage and onward. To identify the Ts4-reactive 63-kDa molecule, we identified NUP62 as the target of Ts4 in 22-dpp testis using liquid chromatography-tandem mass spectrometry analysis. Because NUP62 has be known to play active roles in a variety of cellular processes including mitosis and cell-migration, the bisecting GlcNAc recognized by Ts4 on NUP62 may play a role in regulating the early development of germ cells in male gonadal organs.

Introduction

Fertilization based on the gamete interaction is believed to be a carbohydrate-mediated event (Tulsiani *et al.* 1997, Diekman 2003, Defaus *et al.* 2016). During previous investigation into the pathophysiology of anti-sperm auto (ASA) antibodies (Abs) that affect the fertilization process, we previously generated several ASA-monoclonal Abs (mAbs) from immunologically naïve aged male mice. We targeted molecules in the acrosomal region of epididymal spermatozoa for mAb production (Yoshitake *et al.* 2008a), because sperm molecules having important roles for fertilization are believed to be located in the head region. Among the mAbs established, one (termed Ts4) reacted to glycoproteins in the epididymal sperm head and testicular proteins (Yoshitake *et al.* 2008a). Its epitope is a specific sugar moiety of testicular glycoproteins containing agalacto-biantennary *N*-glycan with bisecting *N*-acetylglucosamine (GlcNAc) carrying fucose residues (Yoshitake *et al.* 2015).

Bisecting GlcNAc is a β1,4-linked GlcNAc residue attached to a β-mannose of the *N*-glycan core, which is an enzymatic production of *N*-acetylglucosaminyltransferase (GnT)-III (Narasimhan *et al.* 1982). For example, GnT-III is reported to add bisecting GlcNAc to epidermal growth factor receptor and adhesion molecules (e.g., $\alpha_5\beta_1$ integrin and E-cadherin), and to alter their functions (Rebbaa *et al.* 1997, Kitada *et al.* 2001, Isaji *et al.* 2004), therefore, the bisecting GlcNAc structures are thought to play a role in biological functions, such as cell growth and adhesion by regulating cell surface glycoproteins. These results indicate that the oligosaccharide (OS) structure also have potential function(s) in the fertilization process. Histochemical analysis using *Phaseolus vulgaris* erythroagglutinin (PHA-E; a lectin known to recognize complex type *N*-glycans containing bisecting GlcNAc (Narasimhan *et al.* 1986)) has shown that bisecting GlcNAc exists in testicular and epididymal germ cells (Lee & Damjanov 1985). However, its biological function in reproductive processes remains incompletely understood.

Our previous studies revealed specific immunoreactivity of Ts4 against reproduction-related regions, including the acrosomal region of epididymal spermatozoa, the plasma membranes of spermatocytes/spermatids within the seminiferous tubules in adult mice, and the early embryo. However, the immunoreactivity was not observed against the ovary or other major somatic organs (Shirai *et al.* 2009, Yoshitake *et al.* 2015). In matured male organs (epididymis and testis), Ts4 immunoreacts against several distinct glycoproteins (Shirai *et al.* 2009). Among these glycoproteins, we identified alpha-*N*-acetylglucosaminidase (Naglu; a degradation enzyme of heparan sulfate) and TEX101 (a germ cell-marker glycoprotein with an essential role in fertilization *in vivo* (Kurita *et al.* 2001, Fujihara *et al.* 2013)) as possessing the Ts4-recognized bisecting GlcNAc in the epididymal spermatozoa and testicular germ cells, respectively, in adults (Yoshitake *et al.* 2008a, Yoshitake *et al.* 2016). In the early embryo, Ts4 reacted against unknown molecules apart from TEX101 (Shirai *et al.* 2009). These results indicate that the core peptide structure of glycoproteins possessing Ts4-reactive bisecting GlcNAc differ among the epididymal spermatozoa, testicular germ cells, and the early embryo. Based on these findings, we speculated that the expression pattern of such glycoproteins may vary across different stages of male germ cell differentiation and proliferation.

Our ultimate goal is to elucidate the biological significance of Ts4-reactive bisecting GlcNAc in the mammalian reproductive system. As an initial step, in the present study we investigated the molecular expression and chemical characteristics of molecules equipped with Ts4-reactive bisecting GlcNAc in male gonads during development. Furthermore, we identified a glycoprotein possessing Ts4-reactive bisecting GlcNAc at an early developmental stage of spermatogenesis using immunoprecipitation combined with liquid chromatography (LC)-tandem mass spectrometry analysis (MS/MS).

Materials and methods

Animals

Female/male ICR mice (3-15-wk-old) were from Sankyo Lab Service (Tokyo, Japan). They were maintained and bred at our animal facilities under 12L:12D conditions, and given free access to food and water. Animal experimental protocols were approved by the ethical committee for laboratory animals (the approved #270026), and all procedures were conducted according to the guide for care and use of laboratory animals, Juntendo University. For mated animals, observation of the vaginal plug was considered as 0 day-postcoitus (dpc), and the day they were born was regarded as 1 day-postpartum (dpp) for neonatal mice.

Antibodies

Ts4 (mouse IgM) was established as previously described (Yoshitake *et al.* 2008a). RP-3 (an anti-rat polymorphonuclear leukocyte mAb, mouse IgM) used for control studies, TES101 (an anti-TEX101 mAb which reacts only under non-reducing conditions, mouse IgG1), 6035 (an anti-TEX101 mAb which reacts under either reducing and non-reducing conditions, mouse IgG2a), 3H9 (an anti-human GPI-80 mAb, used as negative control mAb of TES101, mouse IgG1), and anti-dipeptidase 3 (DPEP3) polyclonal antibody (pAb) (rabbit IgG) were generated and purified as previously reported (Sekiya *et al.* 1989, Suzuki *et al.* 1999, Kurita *et al.* 2001, Yoshitake *et al.* 2008a, Yoshitake *et al.* 2011). Other Abs were from the following companies: negative control mouse and rabbit immunoglobulins (Ig), horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and goat anti-rabbit Ig pAbs (DAKO, Glostrup, Denmark), anti-DDX4/MVH pAb (rabbit IgG) (Abcam, Cambridge, UK), anti-nuclear pore glycoprotein p62 (NUP62) pAb (rabbit IgG) (Proteintech Japan, Tokyo, Japan), anti-CD73 pAb (sheep IgG), and negative control sheep Ig (R and D systems, Minneapolis, MN, USA),

HRP-conjugated goat anti-mouse IgM pAb (Merck Millipore, Billerica, MA, USA), HRP or Alexa Fluor 594-conjugated donkey anti-sheep Ig pAb, Alexa Fluor 488-conjugated goat anti-mouse IgM, and Alexa Fluor 594-conjugated goat anti-rabbit IgG pAbs (Thermo Fisher Scientific, Waltham, MA, USA). Summary of primary antibody details and their dilutions used in immunohistochemistry/Western blot analyses are listed in Supplementary Table 1.

Immunohistochemistry

For organ isolation, mice were anesthetized with 3% (v/v) sevoflurane (Pfizer Japan Inc., Tokyo, Japan) in oxygen, then the total blood was eliminated by flushing the left ventricular lumen with phosphate buffered saline (PBS; pH 7.4) and draining blood from right atrium. The removed fresh organs were fixed with Bouin's solution (Sigma-Aldrich, St. Louis, MO, USA) overnight at 4°C and then embedded in paraffin wax until use. Sections were de-paraffinized in xylene, washed in ethanol and then rehydrated in PBS. After blocking in 4% goat serum or Block Ace (Megmilk Snow Brand Co., Ltd., Sapporo, Japan) for 1 h at room temperature (RT), the specimens were probed with appropriate concentration of primary Abs (Supplementary Table 1) in PBS overnight at 4°C, and then washed three times in PBS containing 0.05% Tween 20 (Nacalai tesque, Kyoto, Japan) (PBST). Subsequently, the sections were incubated with Alexa Fluor 488- or 594-conjugated secondary Abs (2 μg/mL) at RT for 1 h. After rinsing with PBST three times, the sections were counterstained with TO-PRO3 (Thermo Fisher Scientific) for 20 min at RT, washed with PBST three times, and then mounted in Ultramount Aqueous Permanent Mounting Medium (DAKO) on glass microscope slides (Matsunami Glass, Osaka, Japan). The stained-samples were observed under a confocal laser microscope (TCS SP5 AOBS, Leica, Wetzlar, Germany). Control sections received the same treatment with an isotype-matched control Ab.

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Preparation of mouse tissue extracts

Tissues from ICR mice were separated into three fractions, the extracellular (EC), the water-soluble (WS), and the Triton-soluble (TS) fractions, according to the methods essentially as described previously (Yoshitake *et al.* 2008a, Yoshitake *et al.* 2008b, Yoshitake *et al.* 2015). In some cases, to
150 prepare tissue extractions from mice under 29-dpp-old, the tissues were extracted as follows; after homogenizing by 20 strokes with a glass homogenizer in PBS containing 1 × EDTA-free complete inhibitor cocktail (Roche Diagnostics GmbH, Penzberg, Germany), the sample were subsequently extracted by Digital homogenizer for 10 sec, six times. Then Triton X-100 was added to the final concentration of 1%. Following a 20 min incubation, the suspension was ultracentrifuged at 114,000
155 × g for 30 min, and the resultant supernatant was designated as the whole protein (WP) fraction. All subsequent steps were performed on ice. All samples were stored at -80°C until use.

SDS-PAGE and Western blot analysis

The proteins were separated by one-dimensional (1-D) or 2-D SDS-PAGE system, and Western blot
160 analysis was performed according to the published methods ((Yoshitake *et al.* 2011, Yoshitake *et al.* 2015). The transblotted-membrane was blocked in 2% skim milk, then incubated with appropriate concentration of primary Abs (Supplementary Table 1) overnight at 4°C. After washing, the membrane was incubated with HRP-conjugated secondary Abs for 1 h at RT, followed by detection using ECL Western blotting detection system (GE Healthcare Life Sciences, Pittsburgh, PA, USA),
165 then exposed on an X-ray film (BioMax XAR Film, Eastman Kodak Company, Rochester, NY, USA). In some cases, the results from Western blot was digitized into an Apple iMAC computer (Apple Inc.,

Cupertino, CA, USA), and the relative density was traced using ImageJ 1.52 (http://imagej.nih.gov/ij) for relative quantitation.

Immunoprecipitation

Immunoprecipitation using Ts4 and TES101 was performed essentially according to the method described previously (Yoshitake *et al.* 2011, Yoshitake *et al.* 2015). Immunoprecipitation using the anti-CD73 pAb or the anti-NUP62 pAb was performed by Dynabeads Protein G or Protein A (Thermo Fisher Scientific), respectively, according to the manufacturer's instructions. Briefly, 10 µg of the anti-CD73 pAb or negative control sheep Ig was conjugated with 1.5 mg of Dynabeads-Protein G. The antibodies were crosslinked to the beads to avoid co-elution of the antibodies. The testicular extracts were immunoreacted with each antibody-conjugated bead on a rotary shaker for 10 min at RT. In the case of NUP62, 4 µg of the anti-NUP62 pAb or negative control rabbit Ig was immunoreacted with 100 µg of the testicular extract for 12 h at 4°C. Then, 360 µg of Dynabeads-Protein A were added to the mixture and then incubated at 4°C for another 12 h. After washing, the beads were incubated with 3 × SDS sample buffer for 10 min at 70°C. The solubilized proteins were then used as samples for SDS-PAGE.

Silver staining

After the protein solutions were separated by SDS-PAGE, silver staining was performed using PlusOne Silver Staining kit (GE Healthcare Life Sciences) as described previously (Tsukamoto *et al.* 2006). The silver-stained gels were stored in 10% acetic acid solution at 4°C until analyzed.

In-gel digestion

In-gel trypsin digestion of the protein was performed according to the method as described previously (Tsukamoto *et al.* 2006). After digestion, resulting peptide mixtures were extracted with 1% formic acid/50% acetonitrile solution, then concentrated with a speed vacuum to a final volume of 5-10 µl for further analysis by LC-MS/MS.

LC-MS/MS

Proteins were identified by nano-LC-MS/MS using a Triple TOF 5600 system operated with Analyst TF 1.7 software and an Eksigent nano LC system (AB SCIEX, Framingham, MA, USA). The obtained MS data was searched with ProteinPilot 5.0.1 Software (AB SCIEX) using UniProt database (2018_11). A confidence cutoff of 1% false discovery rate was applied for protein identification.

Results

Morphological immunoreactivity of Ts4 at various stages of testicular development

To reveal the immunoreactivity of Ts4 against testis from the embryonic stage to the puberty, we first performed an immunofluorescence study of mouse testes from 14-dpc to maturity. In testes from 14-dpc to 8-dpp, Ts4 showed strong immunoreactivity against cells in the seminiferous tubules, including germ cells (cells that were positive in staining for anti-DDX4/MVH pAb, a marker Ab for the cytoplasm of germ cells (Noce *et al.* 2001)). Ts4 was slightly reactive with the cells outside of the seminiferous tubules, such as interstitial cells (Fig. 1A-D). At this stage, the Ts4-positive signals were predominantly observed inside the distribution of DDX4/MVH, indicating that Ts4 principally immunoreacted with the nuclear membrane of germ cells (Fig. 1A-D). In accordance with testicular development from 15-dpp to 8-wk after birth, the immunoreactivity was restricted to the seminiferous tubules, unlike before 8-dpp. At 15-dpp, Ts4-signals were observed in the cytoplasm of spermatocytes (Fig. 1E). During the developing stage at 22-dpp, Ts4 showed immunoreactivity mainly against plasma membranes, and faint immunoreactivity in the cytoplasm (Fig. 1F). After 29-dpp, Ts4-positive staining was limited to the plasma membranes, including sperm tails (Fig. 1G-I).

Immunoreactive pattern of Ts4 against developing testicular proteins using Western blot analysis

We previously revealed that Ts4 detected approximately 38-, 110-, and 140-kDa bands in sexually mature mouse testicular extracts (Yoshitake *et al.* 2008a), so that we targeted the TS fraction of testis because Ts4-reactive molecules were observed mainly on the plasma membranes of adult testicular germ cells. We reconfirmed this finding in the present study (Fig. 1I). We next investigated whether these same bands were also detected in the developing testes to characterize Ts4-reactive

molecules. In premature testes, we used WP fraction due to their limitation of sample preparation. Immunoreactivity of testicular extracts from 1-dpp to 22-dpp mice gradually increased at the apparent molecular mass of 63-kDa (Fig. 2A). In addition, several bands were observed at the molecular masses greater than 100-kDa in the testicular extracts from 29-dpp mice (Fig. 2A). Conversely, the specific bands at 38-, 110-, and 140-kDa were detected with Ts4 in the TS fraction from 5- to 9-wk-old mouse testes (Fig. 2A), as well as in mature testes (Yoshitake *et al.* 2008a). We previously reported that the 38-kDa band recognized by Ts4 in the mature testis corresponds to TEX101 during the gonadal development (Kurita *et al.* 2001, Takayama *et al.* 2005, Yoshitake *et al.* 2008a, Shirai *et al.* 2009). We examined whether the 63-kDa bands observed in 1-dpp to 29-dpp testicular extracts contained a peptide derived from TEX101. A mAb specific to TEX101 (termed TES101) (Kurita *et al.* 2001) detected a band at an apparent molecular mass of 38-kDa in the testicular extracts from 22-dpp onwards (Fig. 2B). Although non-specific bands larger than 150-kDa were identified in the testicular extracts from 1- to 15-dpp mice (these bands were appeared in the control experiment; data not shown), the 63-kDa band was not detected with TES101 (Fig. 2B). This result indicates that Ts4 has immunoreactivity against a different protein from TEX101 in the immature testicular extracts.

Identification of the sexually immature testicular protein recognized by Ts4

Attempts were then made to identify the 63-kDa molecule detected in the testicular extracts from 1- to 29-dpp mice (Fig. 2A), using immunoprecipitation method combined with LC-MS/MS. After immunoprecipitation with Ts4 using the testicular WP fraction from 22-dpp mice, we confirmed that Ts4 detected a 63-kDa band in the precipitant (Fig. 2C, lane 1). The immunoprecipitant with Ts4 was separated by SDS-PAGE, and visualized by silver. The four specific bands at apparent molecular masses of 67-, 63-, 61-, and 57-kDa were observed among the Ts4-precipitated proteins

(Fig. 2D, lane 1), but not in the control (Fig. 2D, lane 2). Subsequently, we analyzed the precipitated proteins using LC-MS/MS. The silver staining band at the apparent molecular mass of 67-kDa (Fig. 2D, lane 1, band (a)) contained CD73 (UniProt accession number [UPN]-Q61503) and DPEP3 (UPN-Q9DA79) (Table 1). Furthermore, NUP62 (UPN-Q63850) and DPEP3 were detected in the 63-kDa band (Fig. 2D, lane 1, band (b), Table 1; Supplementary Table 2). The 61-kDa band (Fig. 2D, lane 1, band (c)) contained NUP58 (UPN-Q8R332) (Table 1;), which forms a complex with NUP62 (Finlay *et al.* 1991). In the 57-kDa band (Fig. 2D, lane 1, band (d)), only keratins were detected (Table 1). These results suggest that Ts4 may directly bind to NUP62 and DPEP3 detected in the 63-kDa band, whereas the CD73 observed in the 67-kDa band may form complexes with Ts4-reactive molecules.

Confirmation of the molecules identified by LC-MS/MS analysis

Next, we confirmed the results of the proteomic analysis using immunohistochemical and Western blot analyses. Among the proteins detected by LC-MS/MS, DPEP3 has already been identified as a protein that forms complexes with one of the Ts4-immunoreactive molecules, TEX101, in testicular germ cells at maturity (Yoshitake *et al.* 2011). The immunofluorescence study indicated immunoreactivity of anti-DPEP3 pAb with the plasma membrane of germ cells in testes from 22-dpp mice, which was similar to the immunoreactivity of Ts4 (Suppl. Fig. 1A-C). Western blot analysis revealed that DPEP3 existed in the Ts4-immunoprecipitant from testicular extracts of 22-dpp mouse (Suppl. Fig. 1D, lane 1). However, since the anti-DPEP3 pAb used in this experiment is incompatible with the immunoprecipitation assay (Yoshitake *et al.* 2011), we could not independently confirm that DPEP3 was one of the Ts4-reactive molecules in 22-dpp testes.

Using Western blot analysis, we determined that NUP62 was present in the Ts4-immunoprecipitant from 22-dpp mice testicular extracts (Fig. 3A, lane 1), but not in the normal control IgM-precipitated

proteins (Fig. 3A, lane 2), or in the other controls (Fig. 3A, lanes 3 and 4). We examined whether Ts4 showed immunoreactivity against the immunoprecipitant identified with the anti-NUP62 pAb. As expected, the specific 63-kDa band was detected with either anti-NUP62 pAb or Ts4 in the immunoprecipitant (Fig. 3B, lanes 1 and 3). In addition, Western blot analysis of the Ts4-immunoprecipitant separated by 2-D electrophoresis showed that the anti-NUP62 pAb detected a spot at the apparent isoelectric point/molecular mass of pI 5/63 kDa, which merged with the spot observed using Ts4 (Fig. 3C, overlay).

We further investigated the localization of the Ts4-reactive molecules and NUP62 within the testis from 22-dpp mouse using immunohistochemical analysis. However, the commercially available anti-NUP62 pAb in this study was incompatible with immunohistochemical applications (data not shown). As an alternative way to determine the subcellular localization of NUP62, the 22-dpp testes were extracted into EC, WS and TS fractions and subjected to Western blot analysis using anti-NUP62 pAb. The 63-kDa band was detected in all the three fractions (Fig. 4, lanes 2-4), as well as in the WP fraction (Fig. 4, lane 1). The 63-kDa band was also detected with Ts4 in the EC, WS and TS fractions (Fig. 4, lanes 6-8). In addition to the 63-kDa band, Ts4 recognized a 38-kDa band (presumably corresponding to TEX101) (Fig. 4, lane 8), that was not detectable in the same amount of the WP fraction.

Relationship between CD73 and Ts4-reactive molecules in testicular germ cells at 22-dpp

Concerning candidate molecules of Ts4 reactive proteins apart from NUP62, the immunofluorescence study showed that the anti-CD73 pAb was immunoreactive mainly with the plasma membrane of germ cells in testes from 22-dpp mice (Suppl. Fig. 2B), which was similar to the Ts4 immunoreactivity

pattern (Suppl. Fig. 2C). In addition, Western blot analysis revealed that CD73 existed in the Ts4-immunoprecipitant from 22-dpp mice testicular extract (Suppl. Fig. 2D, lane 1), but not in the normal control IgM-precipitated proteins (Suppl. Fig. 2D, lane 2), or in the other controls (Suppl. Fig. 2D, lanes 3 and 4). Taking a bi-directional approach, we examined whether the immunoprecipitant obtained with the anti-CD73 pAb contained both CD73 itself and the molecules reactive to Ts4 in 22-dpp mice testicular extract. The anti-CD73 pAb-immunoprecipitant reacted with the same pAb (Suppl. Fig. 2E, lane 1), but not with Ts4 (Suppl. Fig. 2F, lane 1). This result suggested that although the 22-dpp mice testicular immunoprecipitant with Ts4 contained CD73, it did not contain a detectable level of the Ts4-reactive molecules. These results led us to speculate that CD73 was a member of the molecular complexes containing the Ts4-reactive molecules (e.g., TEX101 and NUP62), but was not a glycoprotein directly recognized by the mAb.

Next, we verified this hypothesis. Western blot analysis showed that the proteins immunoprecipitated with TES101 (an anti-TEX101 mAb, compatible for immunoprecipitation) were recognized by 6035 (a mAb specific to the peptide portion of TEX101 (Yoshitake *et al.* 2008a)) (Suppl. Fig. 3, lane 1), Ts4 (Suppl. Fig. 3, lane 3), the anti-CD73 pAb (Suppl. Fig. 3, lane 5) and the anti-DPEP3 (Suppl. Fig. 3, lane 9), but not by the anti-NUP62 pAb (Suppl. Fig. 3, lane 7). These results indicate that CD73 forms a complex with TEX101 and DPEP3 (one of the molecules associated with TEX101 (Yoshitake *et al.* 2011)), but not with NUP62 in the 22-dpp testis.

Characterization of Ts4-reactive OS chain modified to NUP62 in 22-dpp testis

We previously reported that the reactivity of Ts4 against TEX101 in the adult testis vanished after digestion with *N*-glycanase (an endoglycosidase that releases intact *N*-glycans from glycoproteins by cleaving between the innermost GlcNAc and asparagine residue of *N*-glycosylation sites (Tarentino *et*

315 *al.* 1985)) and β-*N*-acetylglucosaminidase (a highly specific exoglycosidase that catalyzes the hydrolysis of terminal non-reducing GlcNAc residues) (Wong-Madden & Landry 1995)) (Yoshitake *et al.* 2015). Next, we investigated whether these enzymes also abolished the Ts4 reactivity in the 22-dpp testis using Western blot analysis. Unexpectedly, treatment of the 22-dpp testicular extract with *N*-glycanase did not change the reactivity of Ts4 (Fig. 5A, lanes 1 and 2) or the anti-NUP62 pAb
320 (Fig. 5A, lanes 3 and 4). However, the size of band recognized by the anti-CD73 pAb decreased from 67-kDa to 58-kDa after digestion with *N*-glycanase (Fig. 5A. lanes 5, 6). This result suggests that the Ts4-reactive OS chain on NUP62 in the 22-dpp testis did not possess the sensitivity to *N*-glycanase, unlike TEX101 in the adult testis. We also examined the effect of β-*N*-acetylglucosaminidase on the same OS chain. The 63-kDa band detected with Ts4 was completely
325 abrogated after β-*N*-acetylglucosaminidase treatment (Fig. 5B, lanes 1, 2). Furthermore, the same treatment caused a size reduction of the band recognized by the anti-NUP62 pAb from 63-kDa to 59-kDa (and faintly 55-kDa) (Fig. 5B, lanes 3, 4). Under the same conditions, no change in the mobility of the anti-CD73 pAb was observed (Fig. 5B, lanes 5, 6). Collectively, these results indicate that the Ts4-reactive OS chain on NUP62 in the 22-dpp testis contains terminal GlcNAc residues.

330 In addition, we compared to the immunoreactivity of both Ts4 and anti-NUP62 antibody against testicular WP from 1-dpp to 29-dpp (Fig. 6A). During testicular development after the birth, Ts4-immunoreactivity is gradually increased from 1-dpp to 22-dpp and then decreased at 29-dpp, whereas NUP62 expression is constantly increased in parallel with term of after birth (Fig. 6B). These results suggest that the bisecting GlcNAc modification on NUP62 is regulated in the developmental stage.

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Expression of NUP62 and CD73 in the testis of sexually mature mouse

We reported that Ts4 reacts with various kinds of proteins in the adult testis, especially the WS fraction (Yoshitake *et al.* 2008a). Next, we examined whether NUP62 is expressed as a Ts4-reactive molecule in the adult testis, as well as in the sexually developing testis. Western blot analysis showed
340 that 63-kDa bands were detected with both Ts4 and the anti-NUP62 pAb in the Ts4-immunoprecipitated testicular WS fraction of 8-wk-old mouse (Fig. 7A), suggesting that NUP62 exists in the sexually mature testis while possessing the Ts4-reactive OS chain. We further found that CD73 was also associated with TEX101 in the adult testis (Fig. 7B).

Discussion

In the present study, we demonstrated for the first time, that Ts4 exhibits immunoreactivity against the immature mouse testis. However, the pattern of Ts4 reactivity was different from that of the adult testis (Figs. 1, 2). Our previous immunohistochemical analysis of the mature testis showed that Ts4-reactivity was observed mainly on the plasma membrane of germ cells (Yoshitake *et al.* 2008a, Shirai *et al.* 2009), indicating that the Ts4-recognized OS chain binds to membrane glycoproteins. Indeed, TEX101 expressed on cell surfaces as a glycosylphosphatidylinositol-anchored protein (GPI-AP) (Jin *et al.* 2006) was identified as one of the molecules modified by the Ts4-reactive bisected *N*-glycan in the mature testis (Yoshitake *et al.* 2008a). Conversely, positive signals in the testes were found on the nuclear membrane from 14-dpc to 8-dpp, in the cytoplasm at 15-dpp, and mainly on the plasma membrane/faintly in the cytoplasm of germ cells at 22-dpp (Fig. 1A-F). In addition, we showed using Western blot analysis that Ts4 binds to the 63-kDa molecule, a different molecule from TEX101, in the immature testes from mice before 29-dpp (Fig. 2). After 29-dpp, Ts4 exhibits reactivity against several glycoproteins, including TEX101. We previously reported that Naglu was recognized with the mAb in the epididymal spermatozoa (Yoshitake *et al.* 2016). Therefore, these previous and present results indicate that the molecules possessing Ts4-reactive bisecting GlcNAc change in male germ cells during its differentiation and maturation. Using an immunoprecipitation method combined with liquid LC-MS/MS, we identified DPEP3, CD73, and NUP62 as potential glycoproteins modified with the Ts4-recognized bisected *N*-glycan in the immature testis (Table 1, Suppl. Table 2).

DPEP3 belongs to the membrane-bound dipeptidase family (Habib *et al.* 1998), which has an enzymatic activity for the conversion of leukotriene D4 to leukotriene E4 or the cleavage of cystinyl-bis-glycine (Habib *et al.* 2003). Expression of DPEP3 is observed only in the testis (Habib *et al.* 2003); however, its biological function *in vivo* remains unclear, although a recent study reported that

Dpep3-deficient mouse is fertile (Xie *et al.* 2019). We previously reported that DPEP3 was not directly recognized by Ts4 in the mature testis, but that TEX101 was physically associated with DPEP3 (Yoshitake *et al.* 2011). The present study indicates that CD73 also forms a complex with TEX101 in 22-dpp and adult testes (Suppl. Fig. 3), but not possesses Ts4-reactive OS chain (Suppl. Figs. 2 and 3). CD73, otherwise known as ecto-5′-nucleotidase, is an enzyme that dephosphorylates extracellular adenosine 5′-monophosphate (AMP) into adenosine via a purinergic signaling pathway (Zimmermann *et al.* 2012, Regateiro *et al.* 2013). CD73 expression is widely observed in various tissues, such as the brain, heart, lung, liver, kidney, colon, and placenta (Resta *et al.* 1993, Thompson *et al.* 2004), as well as some types of leukocytes, including T cells, neutrophils, monocytes/macrophages, and dendritic cells (Antonioli *et al.* 2013). The expression of CD73 in the testis has also been reported previously (Martin-Satue *et al.* 2010). Concerning CD73 and adenosine in general, many studies have reported that both molecules are key modulators for suppression of the immune response in the tumor microenvironment (Stagg & Smyth 2010, Salmi & Jalkanen 2012, Stagg 2012, Zhang 2012). Interestingly, CD73 exists on cell surfaces as a GPI-AP (Antonioli *et al.* 2013), as do several other molecules related to Ts4, including TEX101, DPEP3, and Ly6k (Jin *et al.* 2006, Yoshitake *et al.* 2008b, Yoshitake *et al.* 2011). At present, GPI-APs are believed to exist on membrane microdomains (known as lipid rafts) containing abundant cholesterol, sphingolipids, and Src-family kinases (Lingwood & Simons 2010). A recent report suggested that each GPI-AP possessing complex and high mannose type of *N*-glycans forms a specific lipid raft (Miyagawa-Yamaguchi *et al.* 2015). The molecules associated with TEX101, including CD73, create cluster-formations in the same lipid raft, and may belong to the molecular group configuring the lipid raft on germ cells in testis.

Among the candidate molecules targeted by Ts4 (Table 1, Suppl. Table 2), data obtained from the present study clearly demonstrated NUP62 as a glycoprotein in immature testis possessing a Ts4 reactive OS chain (Fig. 3). NUP62 was first identified as a component of nuclear pore complexes (NPC) with roles in nucleocytoplasmic transport. This molecule is reported to localize outside the NPC and to be involved in a wide variety of processes apparently unrelated to NPC function, including microtubule regulation in the cytoplasm, centromere and kinetochore function in mitosis, spindle assembly, and cell migration (Yang *et al.* 2006, Hubert *et al.* 2009, Hashizume *et al.* 2013). Unfortunately, the anti-NUP62 pAb used in the present study was incompatible with immunohistochemistry. However, NUP62 is reported to be expressed in the cytoplasm and nucleus of germ cells (Whiley *et al.* 2012).

Although our experimental results allow us to conclude that NUP62 is the Ts4-reactive glycoprotein in the immature testis, the amino acid sequence of mouse NUP62 does not have the typical consensus sequence for *N*-glycosylation, which is asparagine-X (X may be any amino acid other than proline)-serine/threonine (Marshall 1974). In addition to the consensus sequence, atypical motifs for glycosylation have also been reported, including asparagine-X-cysteine (Stenflo & Fernlund 1982, Sun & Zhang 2015, Lowenthal *et al.* 2016), asparagine-X-valine (Sun & Zhang 2015), asparagine-X-glycine (Valliere-Douglass *et al.* 2009), serine/threonine-X-asparagine and glutamine-glycine-threonine (Valliere-Douglass *et al.* 2010). Mouse NUP62 has amino acid motifs for the above sequences (asparagine-X-glycine, five sites; serine/threonine-X-asparagine; two sites, glutamine-glycine-threonine; two sites); therefore, the resistance to *N*-glycanase in Ts4-reactive OS chain in 22-dpp immature testes may be due to an atypical attachment of the OS chain.

We reported that Ts4 exhibits similar reactivity to embryonic stem (ES) cells in immature testes (Shirai *et al.* 2009). Indeed, the shifting distribution of Ts4 reactivity from the nuclear membrane to

cytoplasm in the developing testes (Fig. 1, A-F) is consistent with the previously reported distribution of Ts4 reactivity in the early embryo (Shirai *et al.* 2009).

The reactivity of Ts4 and molecules associated with the mAb during testicular development are summarized in Figure 8. In ES cells, NUP62, CD73, DPEP3, and Ly6k were detected using Western blot analysis (data not shown); however, as previously reported, TEX101 was not detectable, (Shirai *et al.* 2009). ES cells possess a Ts4-reactive OS chain on NUP62 (Suppl. Fig. 4). In immature testicular germ cells, experiments were performed using the WP fraction, which demonstrated the complex formation of TEX101, CD73 and DPEP3. Ly6k, a molecule which forms complex with TEX101 in adult testis (Yoshitake *et al.* 2008b), is also expressed in 22-dpp testis; however, it was not detectable in anti-TEX101 pAb-immunoprecipitant using Western blot analysis (data not shown). Although TEX101 possesses a Ts4-reactive OS chain, the predominant molecule possessing a Ts4-reactive OS chain was determined to be NUP62. After 29-dpp, Ts4 exhibits reactivity against several molecules (Fig. 2A). In adult testis, NUP62, TEX101 and other unknown molecules possess Ts4-reactive OS chains in the WS fraction. CD73, DPEP3, and Ly6k were detected in the WS fraction. However, based on Western blot analysis they do not seem to form complexes in this fraction (data not shown). Using the TS fraction, we showed in the present study that CD73 forms a complex with TEX101 (Fig. 7), and previously reported that TEX101 also forms complexes with DPEP3 and Ly6k (Yoshitake *et al.* 2008b, Yoshitake *et al.* 2011). In cauda epididymal spermatozoa, the Ts4-reactive OS chain is shifted to locate on Naglu (Yoshitake *et al.* 2016).

In summary, we demonstrated that Ts4-reactive molecules changed around 35-dpp, and that NUP62 is the predominant molecule possessing Ts4-reactive bisecting GlcNAc in testis during sexual development. This study provided additional information on the biochemical characteristics of glycoproteins carrying bisecting GlcNAc in relation to mammalian reproductive development.

435 Although we do not yet have direct evidence concerning whether this OS chain detected by Ts4 has biological significance, the experimental results of the present study would contribute to our understanding of the regulation of the early development of testicular germ cells.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Contributorship

RO-S conceived and designed the study, collected data, performed data analysis and interpretation, provided financial support and wrote the article. HY designed and supervised the study, provided financial support and wrote the article. SK, YM, KY and TU collected data and performed data analysis and interpretation. KT, AH, AI, HF and ST analyzed the data, provided financial supports. YA conceived, designed and directed the study, provided financial support and wrote the article. All authors have given approval to the final version of the article.

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Figure legends

Figure 1. Immunohistochemistry of the developing male mouse reproductive organs using Ts4. The sections from 14-dpc (A), 17-dpc (B), 1-dpp (C), 8-dpp (D), 15-dpp (E), 22-dpp (F), 29-dpp (G), 5-wk (H), and 8-wk-old (I) mice were double stained with Ts4 (green) and anti-DDX4/MVH pAb (red), then counterstained with TO-PRO3 (blue). Bars: 100 μm (A, B), 20 μm (C-F), 50 μm (G-I). Solid and dotted lines show the borders of testes and seminiferous tubules, respectively. GC; germ cells, IC; interstitial cells. The locations of immunopositive staining with the Abs in/on the cytoplasm (double arrows), nuclear membranes (single arrows), and plasma membranes (arrowheads) are indicated in the images, respectively. Insets: Higher magnification images of each seminiferous tubule.

Figure 2. Western blot analysis of the testicular proteins during development and biochemical analysis of testicular proteins in the 22-dpp mice co-immunoprecipitated with Ts4. Testicular WP fractions of 1-, 8-, 15-, 22-, and 29-dpp (each 40 μg) and TS fractions of 5-, 7-, and 9-wk-old (each 10 μg) were applied to a 7.5% SDS-PAGE under reducing conditions, electroblotted onto PVDF membrane, and then detected with Ts4 (A). The same samples (each 5 μg) were separated by SDS-PAGE under non-reducing conditions, blotted onto PVDF membrane, and then detected with TES101 (an anti-TEX101 mAb (αTEX101) (Kurita *et al.* 2001)) (B). Closed and open arrowheads indicate the specific bands corresponding to TEX101 (approximately 38-kDa) and unknown proteins (110- and 140-kDa) previously described (Shirai *et al.* 2009), respectively. Expression of β-actin was used as a quantitative internal control. To identify the protein reactive with Ts4 in the premature testis, testicular WP fraction from 22-dpp mice (20 μg) (positive control: p.c.) and the immunoprecipitated

proteins from the WP fraction with either Ts4 or RP-3 (negative control: n.c.) (lane 1 and 2) were applied to each lane of a 7.5% SDS-PAGE gel, and subjected to Western blot analysis under reducing conditions using Ts4 (C) or visualized by silver staining (D). Immunoprecipitation was performed using testicular WP fraction from 22-dpp mice (200 μg) with Ab (15 μg) for Western blot analysis (C). For silver staining, twice-amount of that in Western blot analysis was used (D). Other control experiments were conducted under the same conditions, except for the absence of the tissue extract (-) (C; lane 3 and 4). Arrowhead indicates the specific band observed in Western blot analysis. The specific band observed in the silver staining of the Ts4-immunoprecipitants (D; lane 1, (a)-(d)), and the corresponding gel piece in the immunoprecipitated protein using negative control antibody (D; lane 2) were analyzed by LC-MS/MS. IP (Ab); Ab used for immunoprecipitation. Mr; Molecular mass.

Figure 3. Relation between Ts4-recognized molecules and NUP62 in the testes from 22-dpp mice. Western blot analyses of testicular proteins immunoprecipitated with Ts4 (A) or the anti-NUP62 pAb (αNUP62) (B). Testicular WP fraction (20 μg) (A and B, p.c.) and the immunoprecipitated proteins from the WP fraction with either Ts4 (A, lane 1) or RP-3 (n.c.) (A, lane 2), or either the αNUP62 (B, lanes 1 and 3) or negative control rabbit Ig (n.c.) (B, lanes 2 and 4) were applied to each lane of a 7.5% SDS-PAGE gel under reducing conditions, and subjected to Western blot analysis using the αNUP62 (A; p.c., lanes 1, 2, B; p.c., lanes 1, 2) or Ts4 (B; p.c., lanes 3, 4). Other control experiments were conducted under the same conditions, except for the absence of the tissue extract (-) (A, lanes 3, 4). Arrowhead indicates the specific band detected with αNUP62 pAb. Two-D electrophoresis of Ts4-immunoprecipitant followed by Western blot analysis using the αNUP62 and Ts4 (C). The immunoprecipitated protein from the WP fraction was applied to an

IPGphor strip (7cm; GE Healthcare Life Sciences) and IEF was performed at pH range from 3 to 10. EF was performed as follows: Step and hold from 0 to 300 V over 30 min, Gradient from 300 to 1000 V over 30 min, Gradient from 1000 to 5000 V over 90 min, then hold at 5000 V for 36 min. For the second dimension, the IPGphor strip was placed onto a stacking gel and subjected to 7.5% SDS-PAGE. Testicular WP protein (20 µg) was applied to the left lane of the IPGphor strip for SDS-PAGE. Both the IEF and SDS-PAGE procedures were performed under reducing conditions. The separated proteins were transblotted onto a PVDF membrane, and then probed with αNUP62. After the specific spot was observed, the membrane was incubated with EzReprobe (ATTO Corporation, Tokyo, Japan) to strip the pAb from the membrane. The membrane was re-probed with Ts4. The images detected using the αNUP62 (red) and Ts4 (green) (within dotted lines) were overlaid using Adobe Photoshop Elements 7 (San Jose, CA, USA). Testicular WP fraction of 200 µg and 15 µg of Abs were used for immunoprecipitation for Western blot analysis.

Figure 4. Expression of NUP62 in each fraction of 22-dpp mice testicular extract. Testicular proteins of the WP, EC, WS and TS fraction (each 20 µg) were applied to a 7.5% SDS-PAGE under reducing conditions, electroblotted onto PVDF membrane and then detected with anti-NUP62 pAb (αNUP62) or Ts4.

Figure 5. Glycosylation pattern of the Ts4-reactive molecules in the 22-dpp testis. Western blot analysis of the WP fraction after *N*-glycanase (A) or β-*N*-acetylglucosaminidase (B) digestion. Testicular WP fraction (200 µg) was incubated at 37°C for 24 h with/without 5 mU of *N*-glycanase (100 mU/40 µL) (ProZyme Inc., San Leandro, CA) in a total volume of 50 µL, or for 1 h with/without 20 U of β-*N*-acetylglucosaminidase (4,000 U/mL) (New England Biolabs Inc., Ipswich, MA, USA) in

a total volume of 15 µl. These samples containing 20 µg of substrates were applied and separated by a 7.5% SDS-PAGE gel under reducing conditions, electroblotted onto PVDF membrane, and then detected with Ts4, anti-NUP62 (α-NUP62) or anti-CD73 (αCD73) pAbs. After pull down using PHA-E, the solubilized proteins from the WP fraction were applied and separated by a 7.5% SDS-PAGE gel under reducing conditions, electroblotted onto PVDF membrane, and then detected with the αNUP62 or Ts4 (C) (lanes 2, 4). Testicular WP fraction (20 µg) was applied as positive control (p.c.) (lanes 1, 3).

Figure 6. Comparison of expression profile between NUP62 and Ts4 reactive sugar residues on NUP62. Western blot analysis with Ts4 or anti-NUP62 pAb during testicular development (A). Testicular WP fractions of 1-, 8-, 15-, 22-, and 29-dpp were applied to a 7.5% SDS-PAGE under reducing conditions, electroblotted onto PVDF membrane, and then detected with either Ts4 or anti-NUP62 pAb. Expression of β-actin was used as a quantitative internal control. Densitometry analyses of immunopositive (B). The relative signal intensity for Ts4 (open circle) or anti-NUP62 (closed circle) in panel A are plotted. The immunoreactive density of Ts4 signal from 1-dpp testis was expressed as basic relative value (=1).

Figure 7. Expression of the Ts4-related molecules in the adult testis. Reactivity of the anti-NUP62 pAb (αNUP62) against the Ts4-immunoprecipitant (A). Immunoprecipitants (IP) using 100 µg of WS fraction from 8-wk-old mice with 15 µg of either Ts4 (lanes 1, 3) or RP-3 (n.c.) (lanes 2, 4) were applied and separated by a 7.5% SDS-PAGE gel under reducing conditions, electroblotted onto PVDF membrane, and then detected with Ts4 or αNUP62. Relation between TEX101 and CD73 (B). IP using 50 µg of TS fraction and 7.5 µg of either TES101 (a mAb, compatible for

immunoprecipitation) (lanes 1, 3, 5) or 3H9 (n.c.) (lanes 2, 4, 6) were applied and separated by a 10% SDS-PAGE gel under reducing conditions, electroblotted onto PVDF membrane, and then detected with 6035 (αTEX101), Ts4 or anti-CD73 pAb (αCD73). Arrowhead indicates the specific band detected with each Ab.

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Figure 8. Molecular status of molecules related to Ts4 reactive OS chains during development. In the ES cells, Ts4-reactive OS chain detection is limited to NUP62. During testicular development after birth around by 22-dpp, the OS chain is mainly detected on NUP62, and a trace amount was detected onTEX101 which forms complex with CD73 and DPEP3 (not with Ly6k) in the testicular 700 germ cells. After the initiation of spermiogenesis, several testicular proteins including NUP62 possess the Ts4-reactive OS chain in the germ cells, however, TEX101 forms molecular complex with CD73, DPEP3, and Ly6k on the plasma membrane (PM) of germ cells. Naglu, alpha-*N*-acetylglucosaminidase; GPI, glycosylphosphatidylinositol; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose; OS, oligosaccharide.

Table 1. LC-MS/MS analysis for confirmation of molecules immunoprecipitated with Ts4

Band	Protein	UniProt Accession No.	[a]Cal. Mw	[b]Coverage (%)	[c]Unused ProtScore	Peptide identified
(a)	CD73	Q61503	63,864	7.3	5.59	VVYPAVEGR
						YPFIVTADDGR
						VVQLEVLCTK
						QVPVVQAYAFGK
	DPEP3	Q9DA79	54,247	11.6	3.13	SLGQSPVEVK
						SFYELGVR
						LPNILQR
						LALEQIDLIR
						EQALALMR
(b)	NUP62	Q63850	53,336	3.6	2.04	LAENIDAQLK
						TLIENGEK
	DPEP3	Q9DA79	54,247	3.4	0.06	ELQGVLR
						SLGQSPVEVK
(c)	NUP58	Q8R332	59,445	7.2	2.51	QVQEEISR
						QLLSLAASGLQR
						LETAQELK
						EQYLGYR
						NTLNIDK
(d)	Keratin only					

The results of the proteins only detected in the Ts4-immunoprecipitant are cited.

[a]Cal. Mw: Calculated molecular weight from primary protein sequence without posttranslational modifications.

[b]Coverage (%): The percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence.

[c]Unused ProtScore: A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins. The value was calculated by ProteinPilot 5.0.1 Software.

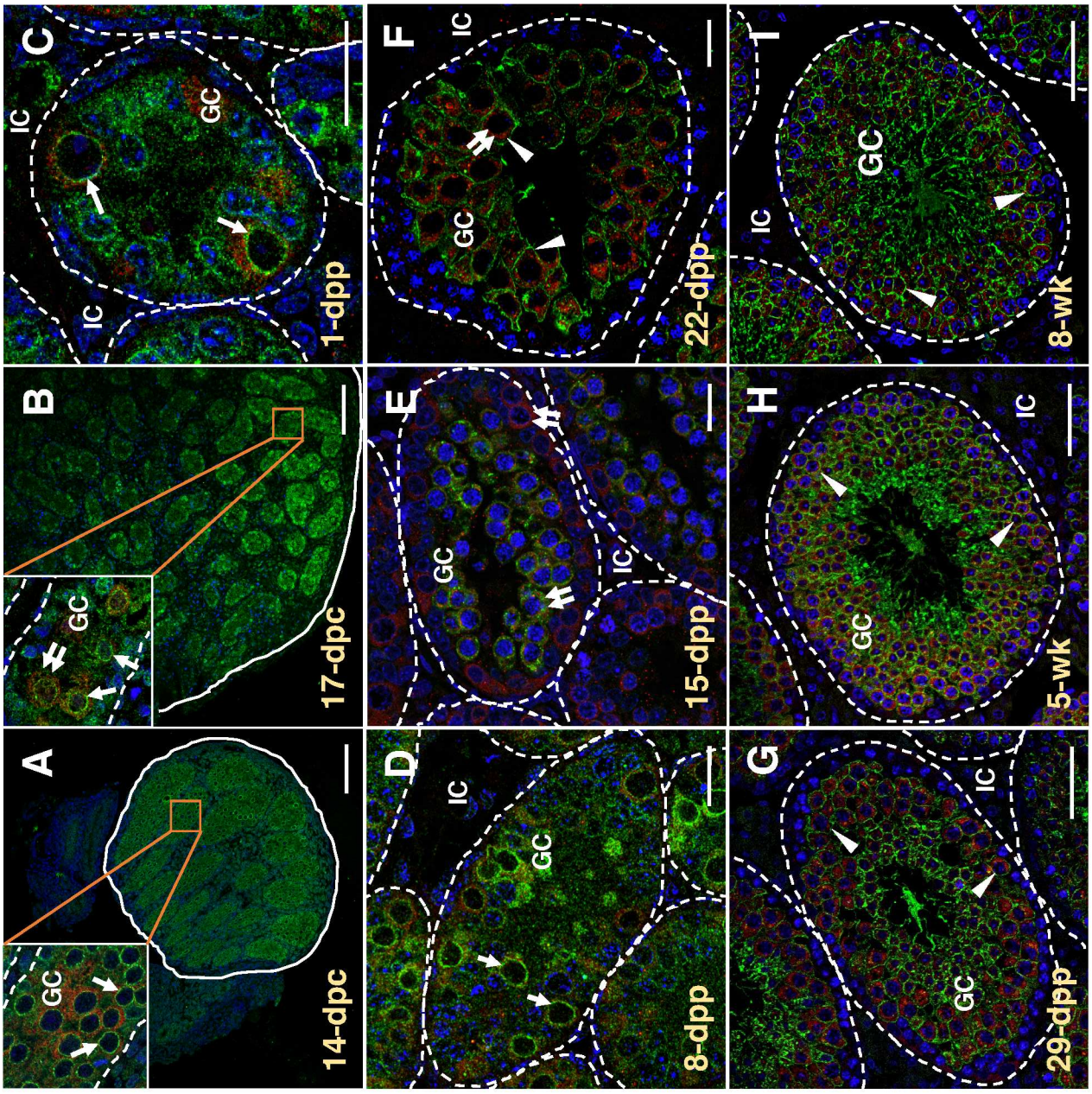


Figure 1. Oda-Sakurai et al.

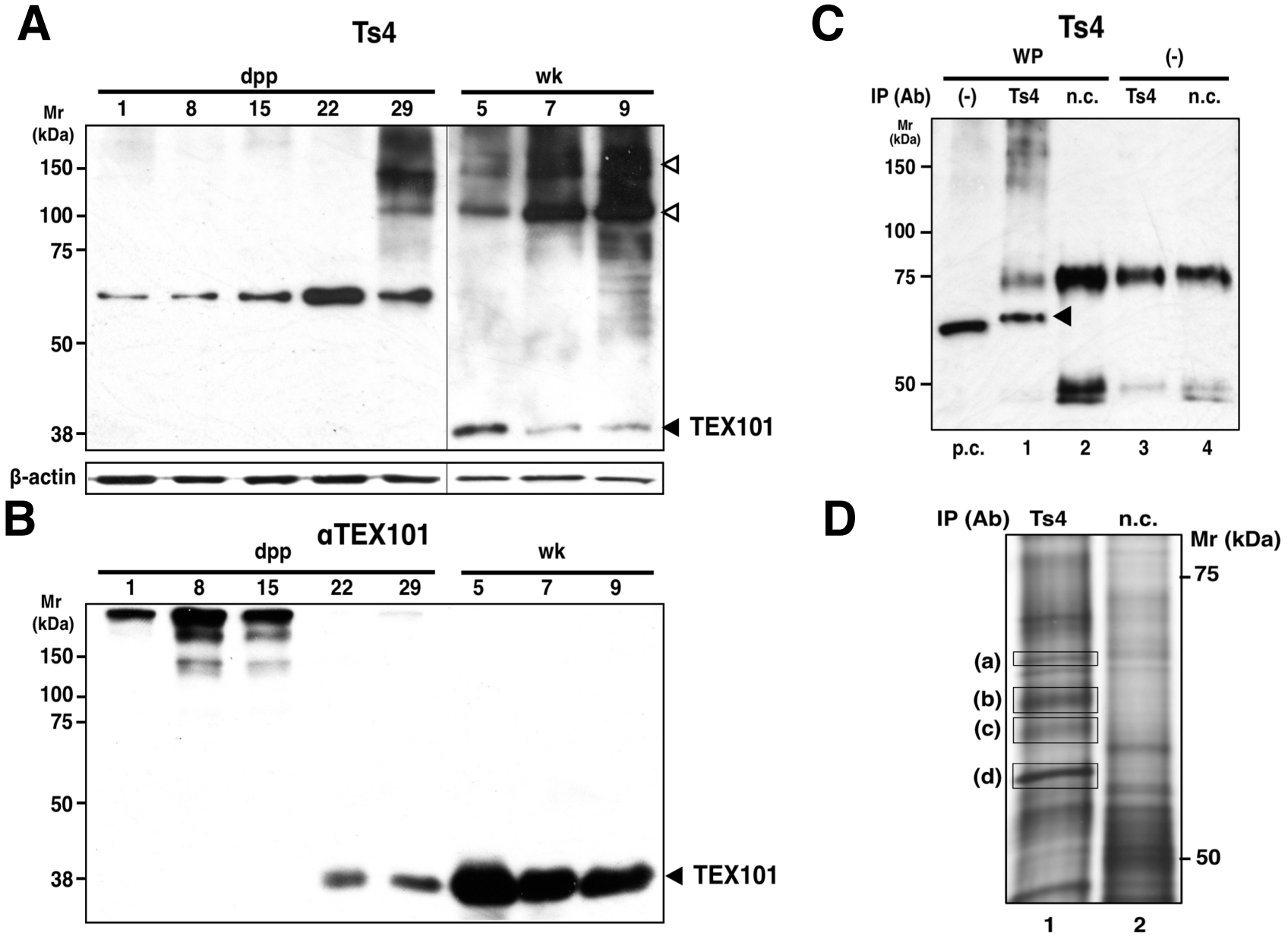


Figure 2. Oda-Sakurai et al.

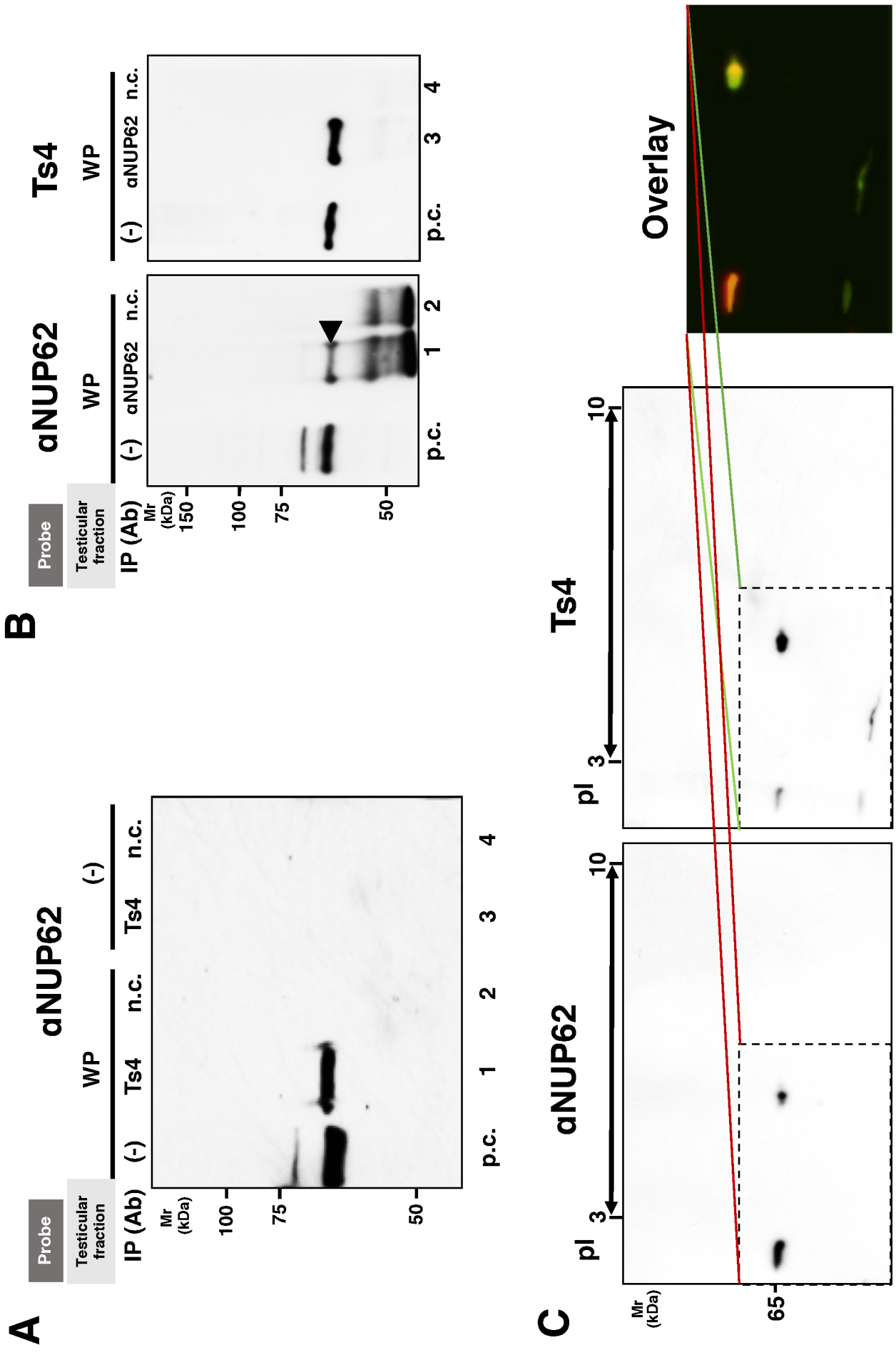
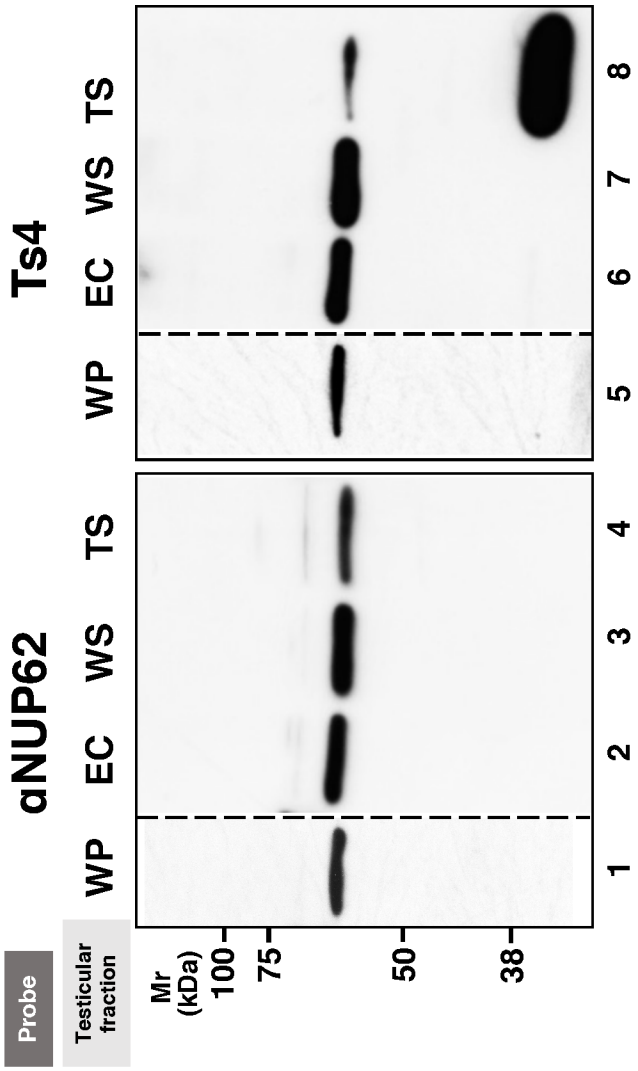


Figure 3. Oda-Sakurai et al.



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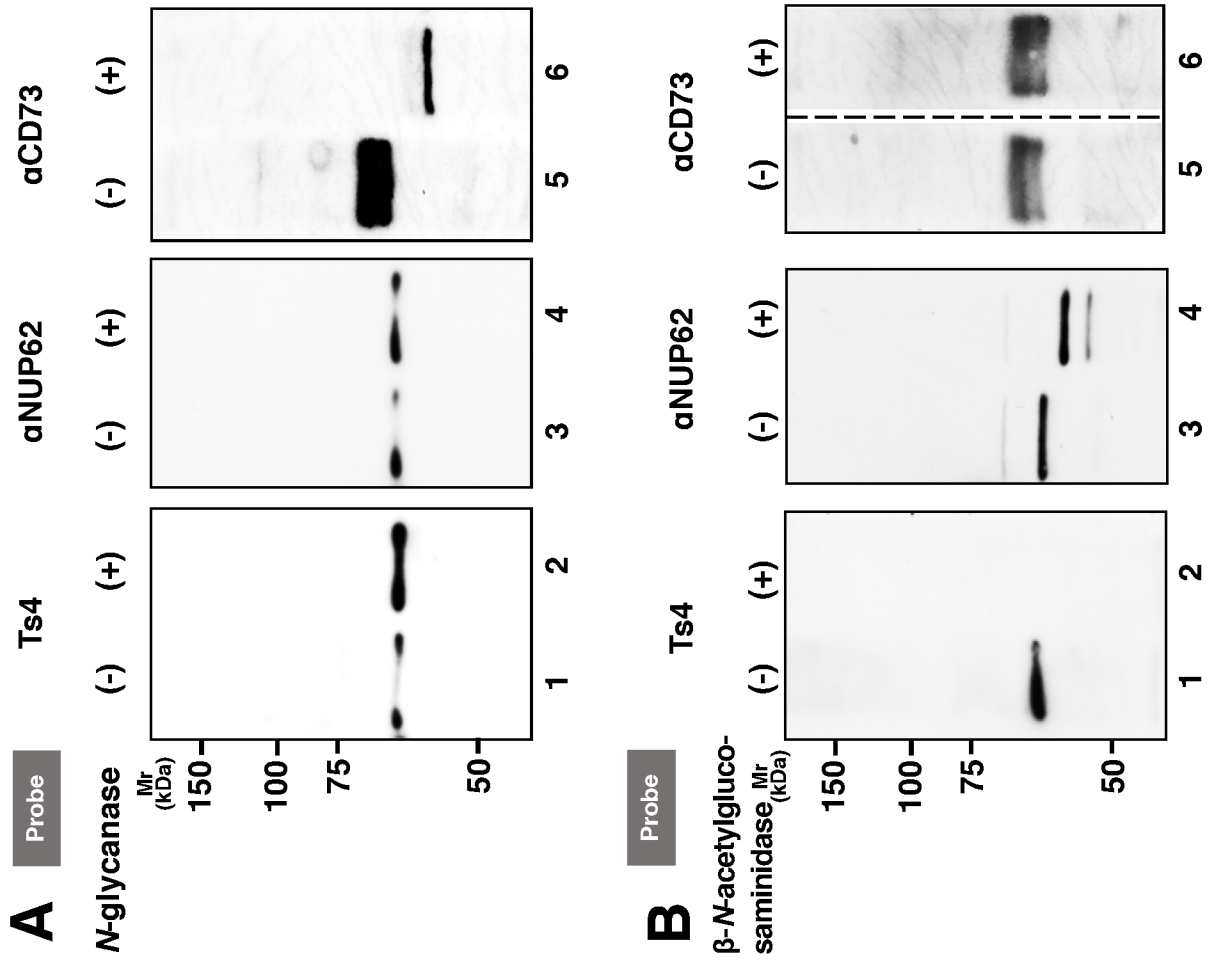

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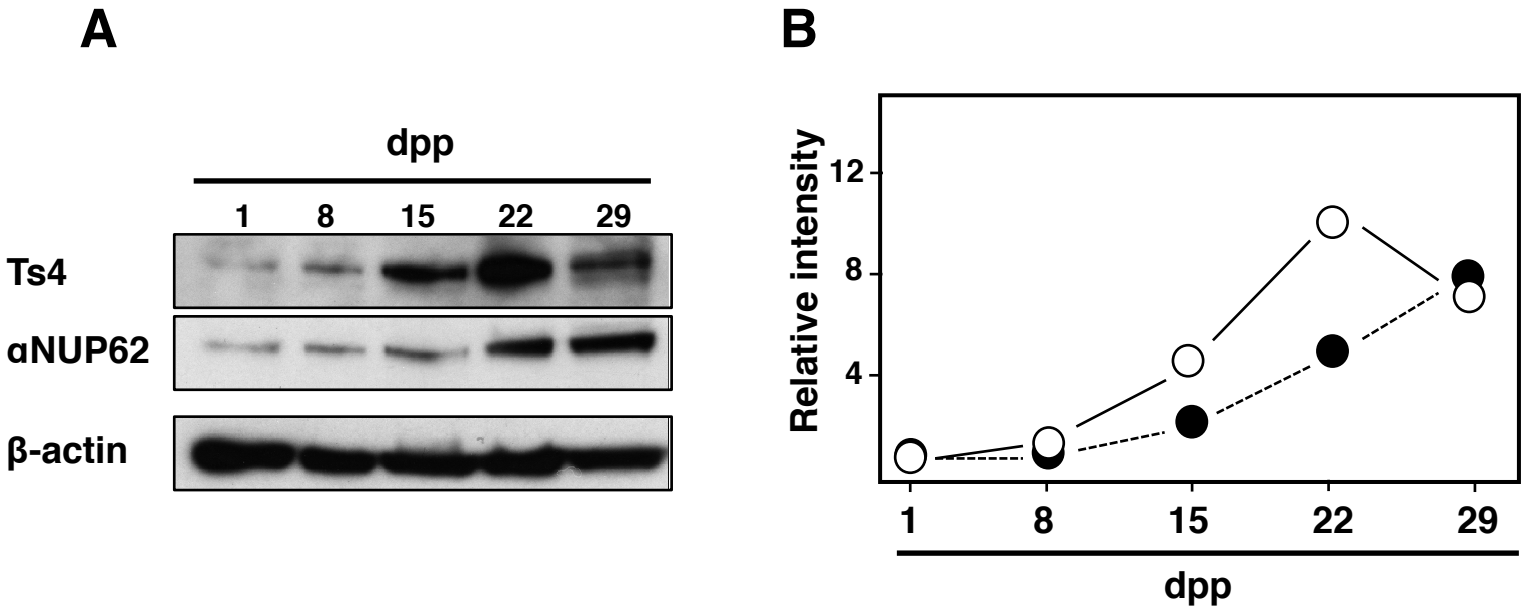

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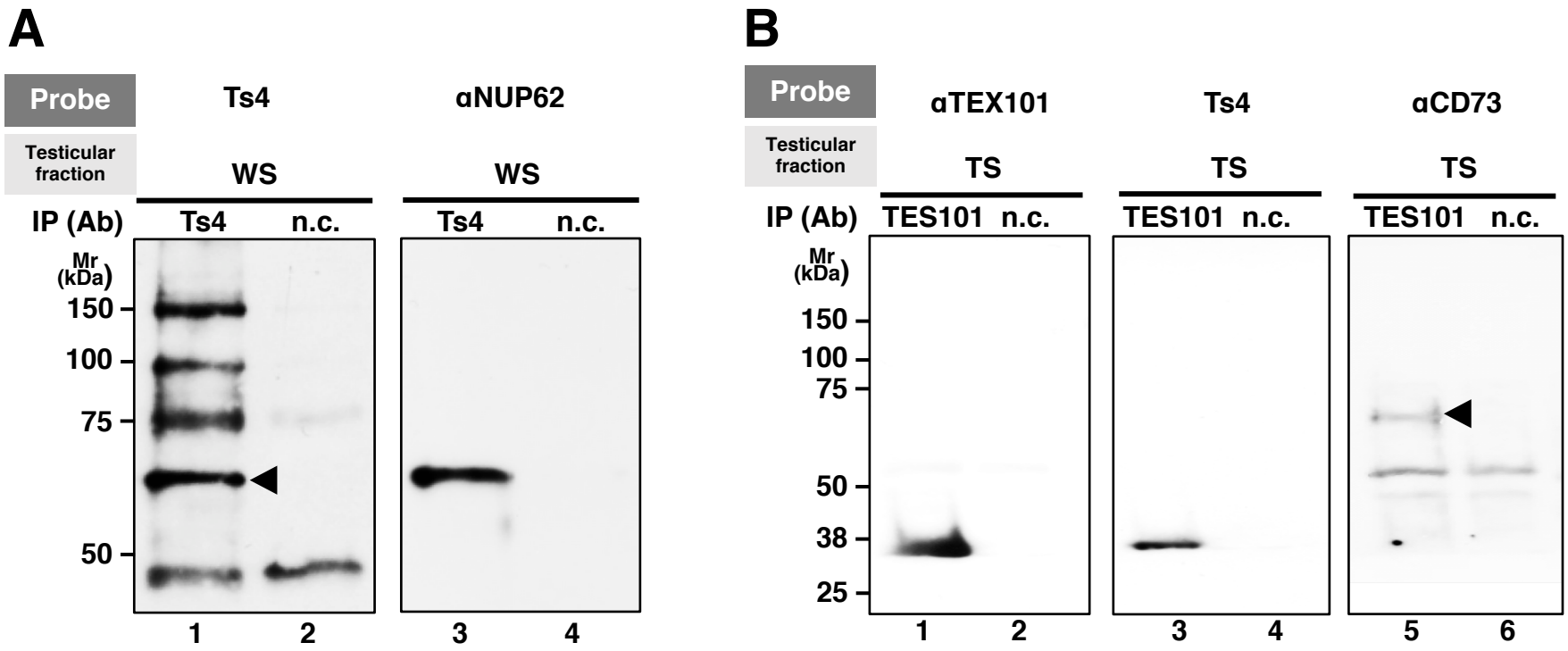


Figure 7. Oda-Sakurai et al.

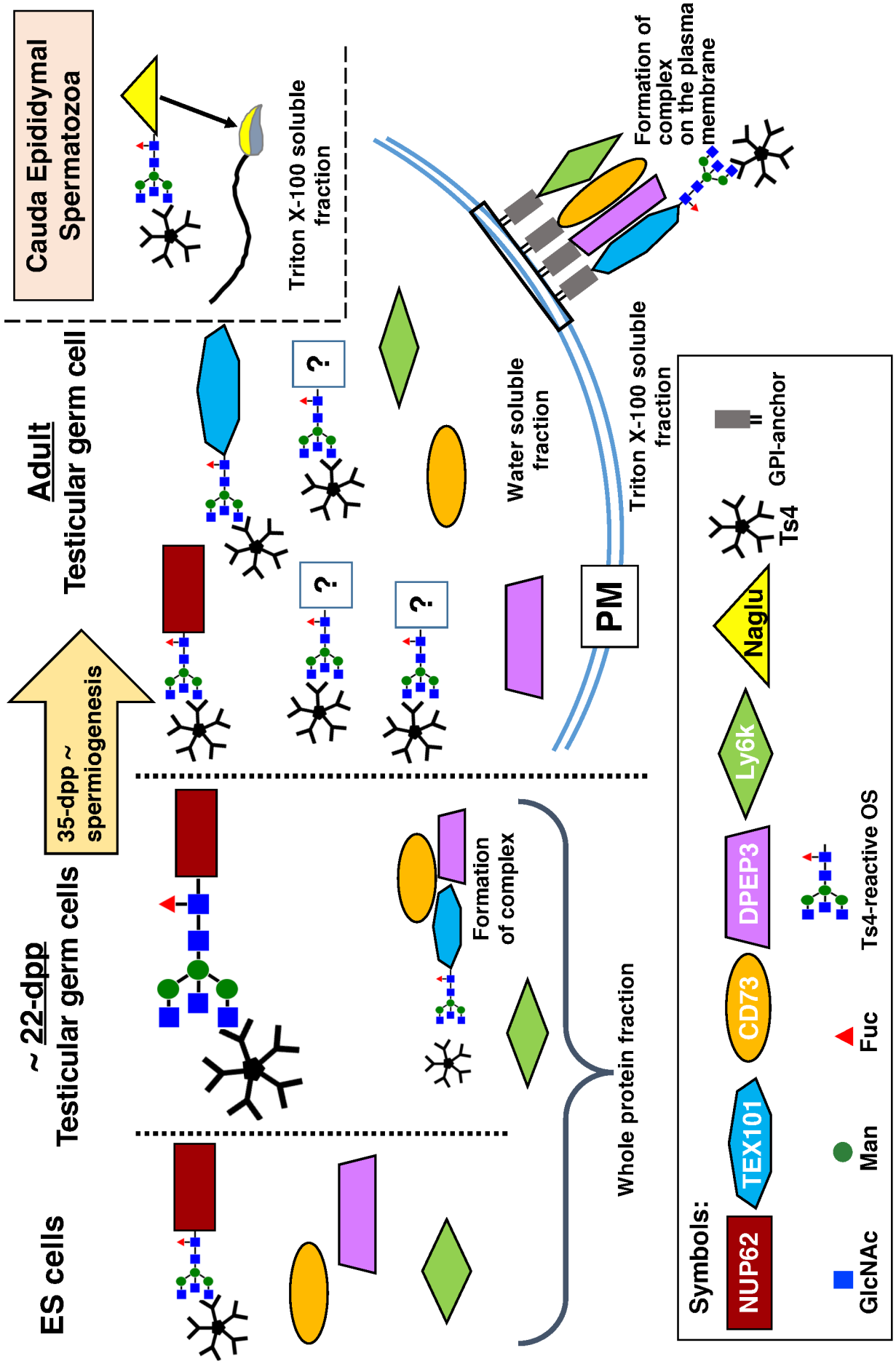


Figure 8. Oda-Sakurai et al.

Supplementary Table 1. Information of primary antibodies used in this study

Primary Ab	(Reference) or Commercial Catalogue#	Subclass (species)	IHC dilution	Western blot dilution
Ts4	(Yoshitake *et al*. 2008a)	IgM (mouse)	5 µg/mL	1 µg /mL
RP-3 (n.c. for Ts4)	(Sekiya *et al*. 1989)	IgM (mouse)	5 µg/mL	1 µg /mL
TES101(anti-TEX101 mAb)	(Kurita *et al*. 2001)	IgG1 (mouse)	n.p.	1 µg /mL
6035 (anti-TEX101 mAb)	(Yoshitake *et al*. 2008a)	IgG2a (mouse)	n.p.	1 µg /mL
3H9 (n.c. for TES101)	(Suzuki *et al*. 1999)	IgG1 (mouse)	n.p.	n.p.
anti-DPEP3 pAb	(Yoshitake *et al*. 2011)	IgG (rabbit)	1/1000 dilution	1/2000 dilution
anti-DDX4/MVH pAb	Abcam #13840	IgG (rabbit)	5 µg/mL	n.p.
anti-CD73 pAb	R and D systems #AF4488	IgG (sheep)	15 µg/mL	1 µg/mL
anti-NUP62 pAb	Proteintech #13916-1-AP	IgG (rabbit)	n.comp.	1/500 dilution

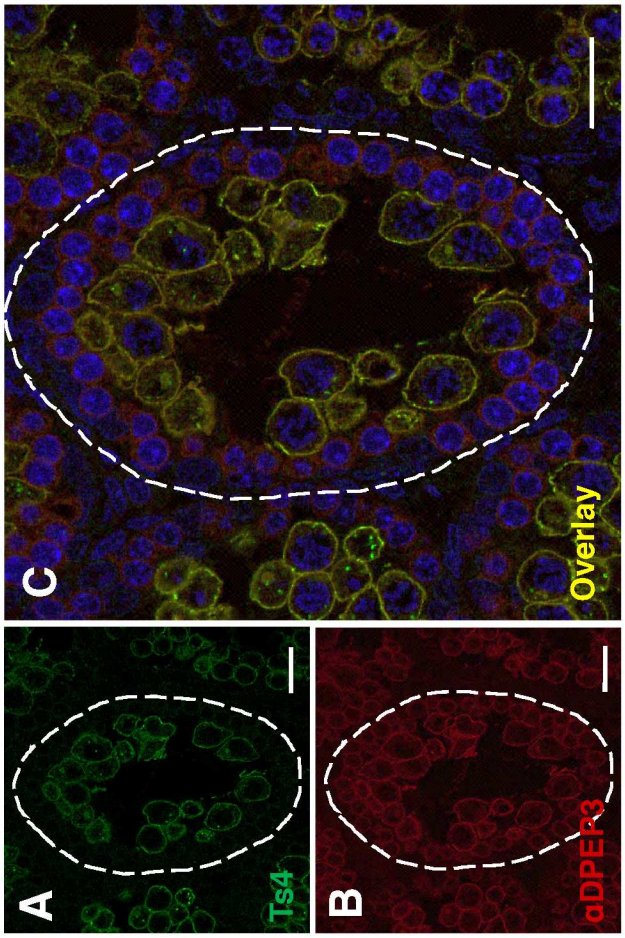
Reference, These Abs were established and characterized by our research group; IHC, immunohistochemistry; n.c., negative control antibody; n.p., not performed; n.comp., not compatible

Supplementary Table 2. LC-MS/MS analysis for confirmation of molecules immunoprecipitated with Ts4 excised from equivalent area showing in Figure 2D-(b)

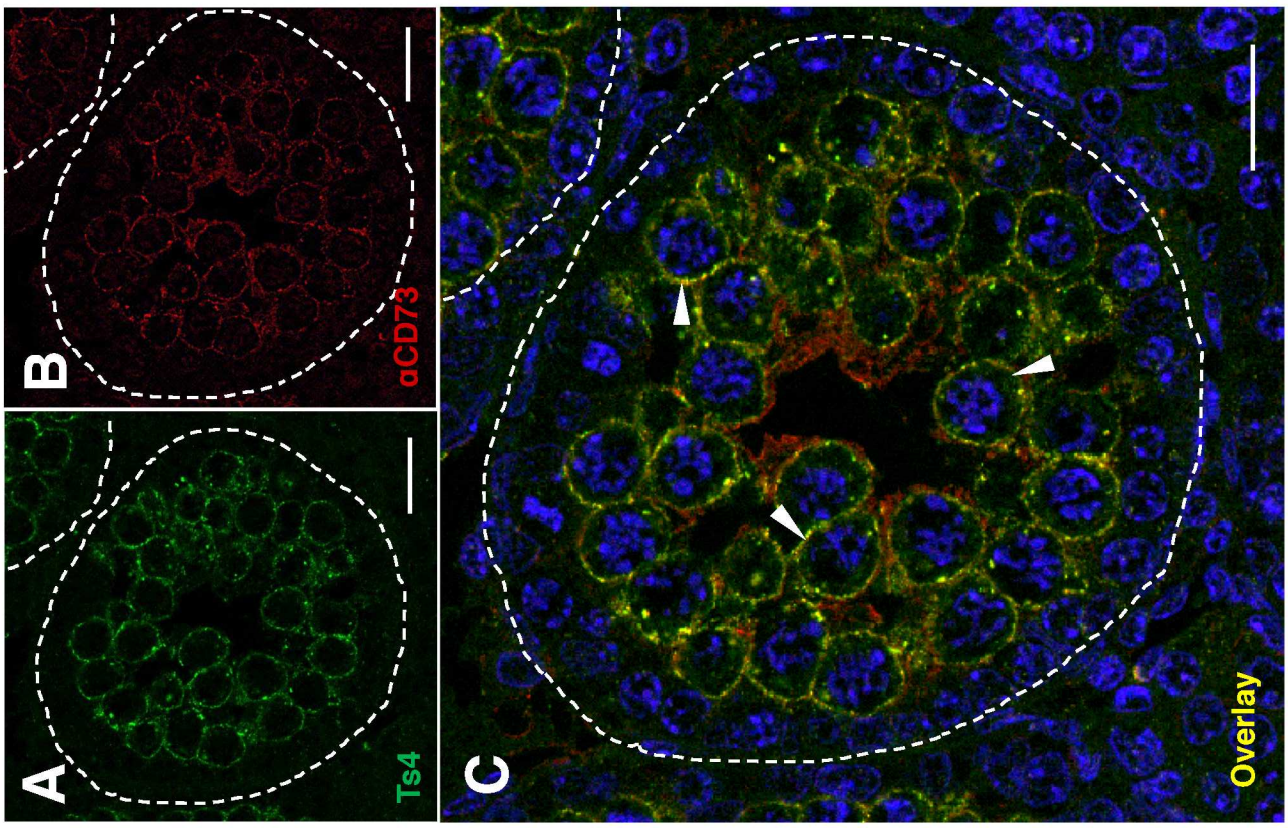
Protein	UniProt Accession No.	[a]Unused ProtScore	Peptide identified	Confidential rate of the peptide detected
NUP62	Q63850	26.39	DIIEHLNMAGGPADTSDPLQQICK	99.0
			ELEDLLSPLEESVK	99.0
			EQSGTIYLQHADEER	99.0
			HFLQQATQVNAWDR	99.0
			ILNAHMDSLQWVDQSSALLQR	99.0
			LAENIDAQLK	99.0
			LDQELDFILSQQK	99.0
			RLDQELDFILSQQK	99.0
			WSLELEDQER	99.0
			EQSGTIYLQHADEEREK	99.0
			SGFNFGGTGAPAGGFTFGTAK	99.0
			PLVSAGPSSVAATALPASSTAAGTATGPAMTYAQLESLINK	99.0
			FNFGGTGAPAGGFTFGTAK	99.0
			ELEDLLSPLEESVKEQSGTIYLQHADEER	98.4
			EQSGTIYLQHADEER	88.9
			TLIENGEK	71.6
NUP58	Q8R332	6.0	ILVQQFEVQLQQYR	99.0
			IYQTFVALAAQLQSIHENVK	99.0
			MFLGDAVDVFEAR	99.0
DPEP3	Q9DA79	2.0	LALEQIDLIR	99.0

The results of the proteins only detected in the Ts4-immunoprecipitant are cited.

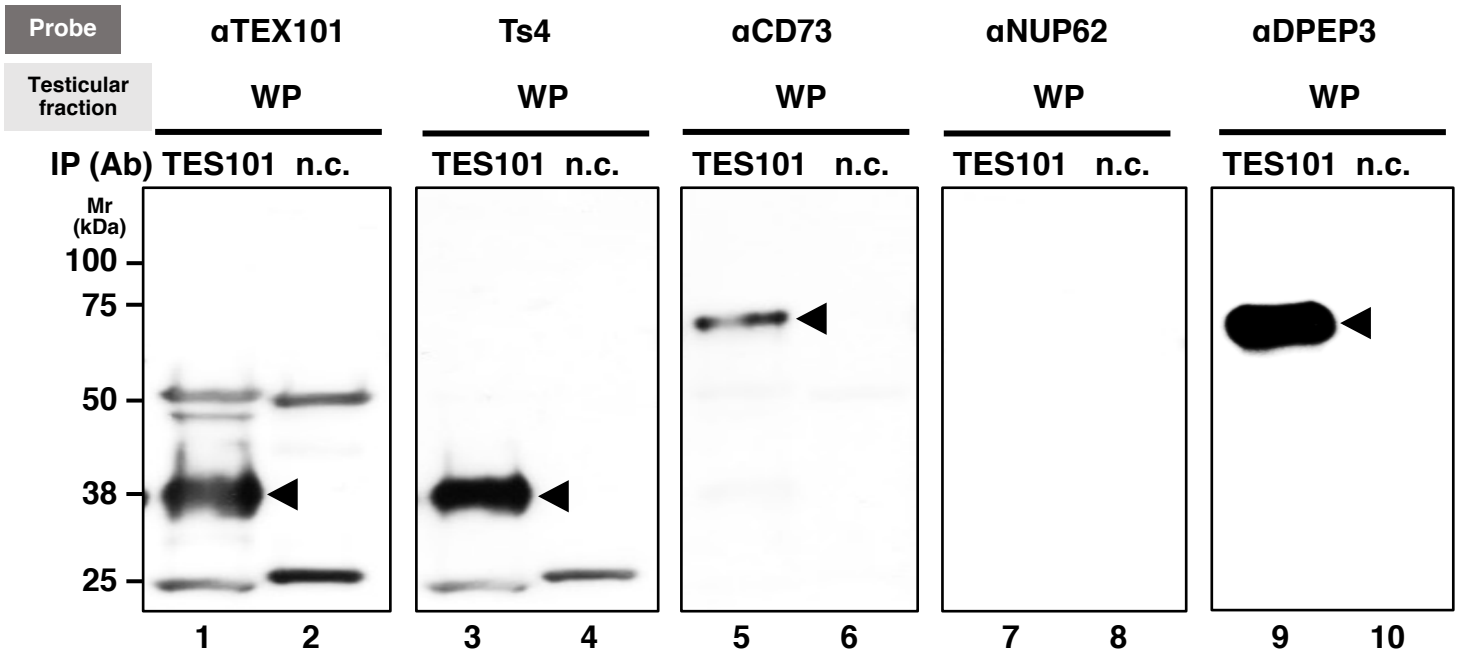
[a]Unused ProtScore: A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins. The value was calculated by ProteinPilot 5.0.1 Software.



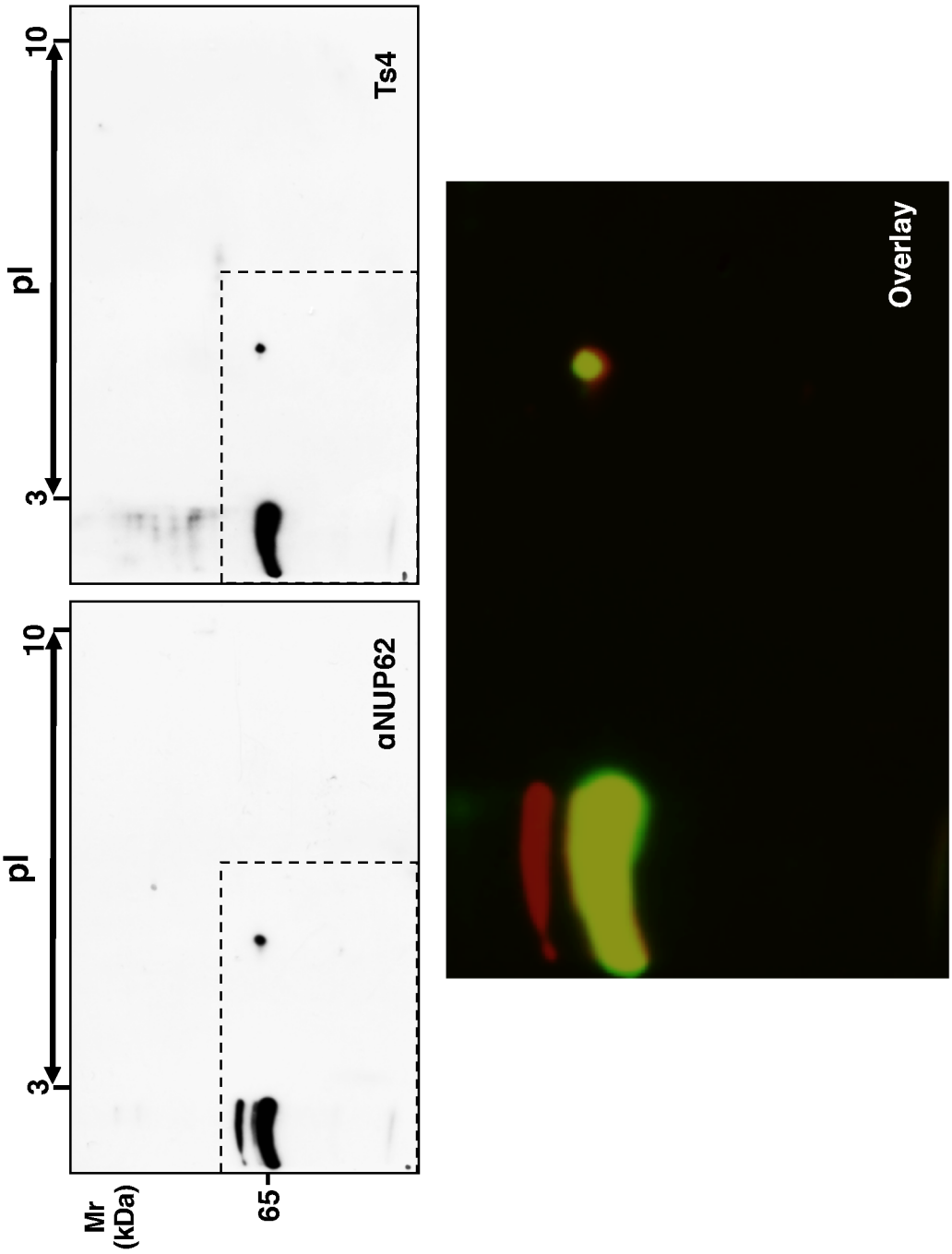
Supplementary Figure 1. Oda-Sakurai et al.



Supplementary Figure 2. Oda-Sakurai et al.



Supplementary Figure 3. Oda-Sakurai et al.



Supplementary Figure 4. Oda-Sakurai et al.

Legends for Supplemental Figures

Supplementary Figure 1. Characterization of DPEP3 in the testis from 22-dpp mice. Immunohistochemical studies of the Ts4-reactive molecule and DPEP3 in the 22-dpp mice testis (A-C). The specimens were treated with these Abs, and then an Alexa Fluor 488- or 594-conjugated secondary antibody was used for detection of the primary antibodies. Green: Ts4 (A), red: anti-DPEP3 pAb (αDPEP3) (B). Overlay image of A and B (C). The samples were counterstained with TO-PRO3 (blue). Bars: 20 μm. White dotted lines indicate the borders of seminiferous tubule. Western blot analysis of Ts4-immunoprecipitant using the anti-DPEP3 pAb (D). The testicular WP fraction of 2.5 μg (p.c.) and immunoprecipitants with either Ts4 or RP-3 using the same fraction (lanes 1 and 2) were applied and separated by a 7.5% SDS-PAGE gel under reducing conditions. Control experiments were conducted under the same conditions, except for the absence of the tissue extract (-) (lanes 3 and 4).

Supplementary Figure 2. Expression of CD73 in the testes from 22-dpp mice. Immunolocalization of Ts4 and the anti-CD73 pAb (αCD73) in the 22-dpp mice testis (A-C). The specimens were treated with these Abs, and then an Alexa Fluor 488- or 594-conjugated secondary antibody was used for detection of the primary Abs. Green: Ts4 (A), red: αCD73 (B). Overlay image of A and B (C). The sample was counterstained with TO-PRO3 (blue). Bars: 20 μm. Dotted lines: the border of seminiferous tubule. Arrowheads: plasma membranes of germ cells in the seminiferous tubule. Western blot analysis of Ts4-immunoprecipitant detected by αCD73 (D). Testicular WP fraction (20 μg) (p.c.) and immunoprecipitants (IP) using 200 μg of WP fraction with either Ts4 or RP-3 (n.c.) (each 15 μg) (lane 1 and 2) were applied and separated by a 7.5% SDS-PAGE

gel under reducing conditions. Control experiments were conducted under the same conditions, except for the absence of the tissue extract (-) (lane 3 and 4). Western blot analysis of the αCD73-immunoprecipitant detected by the same pAb (E) or Ts4 (F). Testicular WP fraction (20 μg) (p.c.) and immunoprecipitants (IP) using 200 μg of WP fraction with either αCD73 or negative control sheep Ig (n.c.) (each 10 μg) were applied to each lane of a 7.5% SDS-PAGE gel (lane 1 and 2) and separated under reducing conditions. Control experiments were conducted under the same conditions, except for the absence of the tissue extract (-) (lane 3 and 4).

Supplementary Figure 3. Western blot analysis of the 22-dpp testicular proteins immunoprecipitated with anti-TEX101 mAb. Immunoprecipitants (IP) using 100 μg of WP fraction with either TES101 (an anti-TEX101 mAb, compatible for IP) or 3H9 (n.c.) (each 15 μg) were applied to each lane of a 7.5% SDS-PAGE gel under reducing conditions, electroblotted onto PVDF membrane, and then detected with 6035 (the anti-TEX101 mAb; αTEX101) (lanes 1, 2), Ts4 (lane 3, 4), the anti-CD73 pAb (αCD73, lanes 5, 6), the anti-NUP62 (αNUP62, lanes 7, 8), or the anti-DPEP3 pAb (αDPEP3, lanes 9, 10). Arrowheads indicate the specific immunoreactive bands observed.

Supplementary Figure 4. Western blot analysis after 2-D electrophoresis of ES cells lysate immunoprecipited with Ts4. The cell pellet of mouse ES cells (a kind gift from Dr. Yoichi Shinkai (RIKEN, Saitama, Japan)) were suspended in buffer A and extracted by Sonifier ultrasound homogenizer for 10 sec, six times. After Triton X-100 was added to the final concentration of 1%, the suspension was incubated for 20 min on ice, and then centrifuged at 10,000 x g for 5 min. The resultant supernatant (ES cell lysate) (200 μg) were immunoprecipitated using Ts4 (30 μg), then applied to an IPGphor strip (7cm; GE Healthcare Life Sciences), and IEF was performed at pH range

from 3 to 10. EF and the following Western blot analysis with anti-NUP62 pAb (αNUP62) or Ts4 were performed as described in the legend for Figure. 3. ES cell lysate (50 μg) was applied to the left lane of the IPGphor strip for SDS-PAGE. The images detected using αNUP62 and Ts4 (within dotted lines) were overlaid using Adobe Photoshop Elements 7 with Ts4 (green) and αNUP62 pAb (red).