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Molecules and Cells

Centrobin/Nip2 Expression *In Vivo* Suggests Its Involvement in Cell Proliferation

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Centrobin/Nip2 was initially identified as a centrosome protein that is critical for centrosome duplication and spindle assembly. In the present study, we determined the expression and subcellular localization of centrobin in selected mouse tissues. Immunoblot analysis revealed that the centrobin-specific band of 100 kDa was detected in all tissues tested but most abundantly in the thymus, spleen and testis. In the testis, centrobin was localized at the centrosomes of spermatocytes and early round spermatids, but no specific signal was detected in late round spermatids and elongated spermatids. Our results also revealed that the centrosome duplication occurs at interphase of the second meiotic division of the mouse male germ cells. The centrobin protein was more abundant in the mitotically active ovarian follicular cells and thymic cortex cells than in non-proliferating corpus luteal cells and thymic medullary cells. The expression pattern of centrobin suggests that the biological functions of centrobin are related to cell proliferation. Consistent with the proposal, we observed reduction of the centrobin levels when NIH3T3 became quiescent in the serum-starved culture conditions. However, a residual amount of centrobin was also detected at the centrosomes of the resting cells, suggesting its role for maintaining integrity of the centrosome, especially of the daughter centriole in the cells.

INTRODUCTION

The centrosome is composed of a pair of centrioles surrounded by the pericentriolar material (PCM). Two centrioles are not identical and can be designated as mother and daughter centrioles, based on their age and morphology. The mother centriole has functioned as a template to produce the daughter centriole in the previous cell cycle. The mother centriole includes appendages to which the nucleated microtubules are anchored while the daughter centriole may be functionally dormant (Piel et al., 2000). PCM consists of amorphous protein matrix that supports centrioles as well as the attached microtubules (reviewed by Blagden and Glover, 2003). The centrosomes are present in most animal cells, but their structure and biological functions differ according to the physiological status of the cells, especially between proliferating and post-mitotic differentiated cells (reviewed by Bornens, 2002).

The centrosomes in most cells function as the main microtubule organizing centers where the minus ends of the microtubules are concentrated. Therefore, the centrosome is engaged in a number of cellular processes that are linked to the cellular microtubule network, such as cell motility, polarity, morphology and intracellular cargo transport. During mitosis, the centrosome functions as a spindle pole, pulling a set of the chromosomes into the daughter cell. The centrosome is also required for formation of cilia and flagella (reviewed by Bettencourt-Dias and Carvalho-Santos, 2008). Finally, the centrosome is a place for initiation of specific cellular functions. For example, the centrosomal Cdk1/cyclin B1 complex is activated as an initial step for mitotic entry, and the centrosomal Chk1 is believed to prevent premature activation of the Cdk1 activity at the centrosome (Jackman et al., 2003; Krämer et al., 2004).

A proteomic analysis revealed that the human centrosome consisted of several hundred kinds of proteins (Andersen et al., 2003). In order to elucidate centrosome functions at the molecular level, we and others have identified and characterized a selected number of the centrosome proteins. Centrosomal centrobin/Nip2 is located in the daughter centriole specifically (Zou et al., 2005). Centrobin was also detected in association with a stable microtubule network of cytoplasm (Jeong et al., 2007). The *centrobin* knockdown resulted in centrosomes with one or no centriole, demonstrating that centrobin is required for centrosome duplication (Zou et al., 2005). The microtubule nucleation and/or anchoring activity of the centrosome was also reduced significantly in the *centrobin*-suppressed cells (Jeong et al., 2007). As a result, cell shrinkage and spindle assembly defects were observed in the *centrobin*-suppressed cells (Jeong et al., 2007). Since overexpression of *centrobin* induced microtubule bundling, it was proposed that centrobin stabilizes the nascent microtubule (Jeong et al., 2007). In order to give clues on centrobin functions in diverse cell types, we observed centrobin expression in selected mouse tissues.

MATERIALS AND METHODS

Animals

Wild type and adult ICR mice were used for tissue preparation. A mouse was used for each of immunoblot, squash, and immunohistochemistry sample. At least three independent ex-

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periments were carried out for immunostaining results. All experiments were carried out in compliance with animal policies of Seoul National University (SNU-050519-4).

Immunoblot analysis

Protein samples were solubilized in the Laemmli sample buffer, resolved by 8% SDS-PAGE, and blotted onto a nitrocellulose membrane. The membrane was blocked by soaking in 0.3% TBST (Tris-buffered saline with 0.3% Triton X-100) with 5% non-fat milk for 30 min, incubated for 1 h with the primary antibody in the blocking solution. The membrane was then washed three times with 0.3% TBST, incubated with a secondary antibody conjugated with horse-radish peroxidase (HRP) for 30 min, and washed 4 times with 0.3% TBST. The signal was detected with the ECL Western blotting detection reagents. The centrobin antibody that had been described previously (Jeong et al., 2007) was diluted to 1:50, the γ-tubulin (Sigma) and PCNA (DAKO) antibodies were diluted to 1:1000, and the secondary antibody conjugated with mouse- or rabbit-HRP (Sigma) was diluted to 1:10000.

Isolation of the testicular germ cells: a squashing method

Squashes of testicular cells were carried out as described (Saunders et al., 2003). In brief, the mouse testis was dissected, placed into a small Petri-dish containing PBS, and the tunica albuginea was removed. The testis was teased apart gently with a blade. The PBS was removed and the sample was fixed with 2% paraformaldehyde in 0.05% PBST (phosphate-buffered saline with 0.05% Triton X-100) at room temperature for 10 min. The fixed sample was placed on a slide glass and squashed by placing a cover slip and pressing it down gently. The slides were immersed in liquid nitrogen and stored at -70°C. For immunostaining, the slides were washed three times with PBS, pre-incubated with a blocking solution (PBST with 3% normal goat serum) for 15 min, incubated with primary antibodies for 1 h at room temperature, washed five times with 0.1% PBST, incubated with secondary antibodies conjugated with FITC or TRITC (Jackson Immunoresearch) for 30 min, washed 5 times with 0.1% PBST, and incubated with a DAPI solution. The purified NIP2 antibody and the γ-tubulin antibody were diluted to 1:100. The secondary antibodies were diluted to 1:100.

Immunohistochemistry

Mouse tissue samples were fixed in 4% buffered paraformaldehyde, embedded in paraffin wax, and processed as described previously (Rhee and Wolgemuth, 1997). In brief, after deparaffinization, slides were boiled in 20mM Tris-HCl buffer, pH 9.0, (Yamashita and Okada, 2005) for 15 min and washed extensively with H_2O. The slides were pre-incubated with the blocking solution (PBST with 3% normal goat serum) for 15 min at room temperature and incubated with the primary antibodies in a humidified chamber for 1 h at room temperature. The slides were then washed five times with 0.1% PBST, incubated with either FITC- or TRITC-conjugated secondary antibodies for 30 min, washed five times with 0.1% PBST and stained with DAPI. The slides were observed under a fluorescence microscope with a CCD (Qicam fast 1394; Qimaging) camera. After taking pictures, the coverslip on the slide was taken off using acetone and the slides were washed with absolute ethanol vigorously. The slides were hydrated and stained with hematoxylin. Pictures from immunofluorescence and hematoxylin staining were compared to find the identical tubules in Figs. 5 and 6.

Cell culture and immunofluorescence

The NIH3T3 cells were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum. For serum starvation, the cells were grown in DMEM with 0.1% FBS for 6 d. For immunofluorescence staining, the cells were seeded on a coverslip coated with poly-L-lysine, fixed in a cold methanol (-20°C) for 10 min, washed three times with 0.1% PBST, blocked with 10% normal goat serum, incubated with primary antibodies, washed three times with 0.1% PBST, incubated with FITC- or TRITC-conjugated secondary antibodies, washed three times with 0.1% PBST, and incubated with DAPI solution.

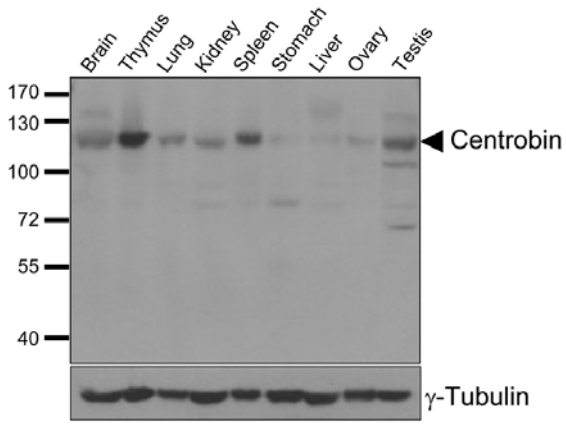

Fig. 1. Immunoblot analysis of centrobin in selected mouse tissues. Thirty-microgram proteins from indicated tissue samples were used for the analysis. The blot was probed with the centrobin antibody. γ-Tubulin was detected as a loading control.

RESULTS

Immunoblot analysis of centrobin in selected mouse tissues

Immunoblot analysis was carried out to determine *centrobin* expression in selected mouse tissues at the protein levels. The mouse centrobin protein of 100 kDa shares 82% identity with the human centrobin protein. The centrobin-specific band was detected in all tissues tested but most abundantly in the thymus, spleen and testis (Fig. 1). This result is consistent with a previous study in which the *centrobin* mRNA was detected in all tissues tested and most abundantly in the testis (Zou et al., 2005).

Reduction of centrobin expression in the serum-starved NIH3T3 cells

In order to examine a correlation of centrobin expression with the cell proliferation activity, we determined its expression in a mouse fibroblast cell line with a reduced proliferation activity. When NIH3T3 cells were cultured in a medium with 0.1% serum, they became quiescent as indicated by reduced PCNA levels (Fig. 2A). We observed that the cellular centrobin level was also reduced significantly in the quiescent cells (Fig. 2A). The immunostaining of the NIH3T3 cells revealed that centrobin is located at the centrosome (Fig. 2B). The centrobin signal overlapped with a fraction of the two γ-tubulin signals, consistent with the previous report in which centrobin is daughter-centriole-specific (Zou et al., 2005). The centrobin signals in the serum-starved cells were reduced significantly in comparison to the γ-tubulin signals (Fig. 2B). These results indicate that the cellular centrobin levels depend on the proliferation activity of the cells.

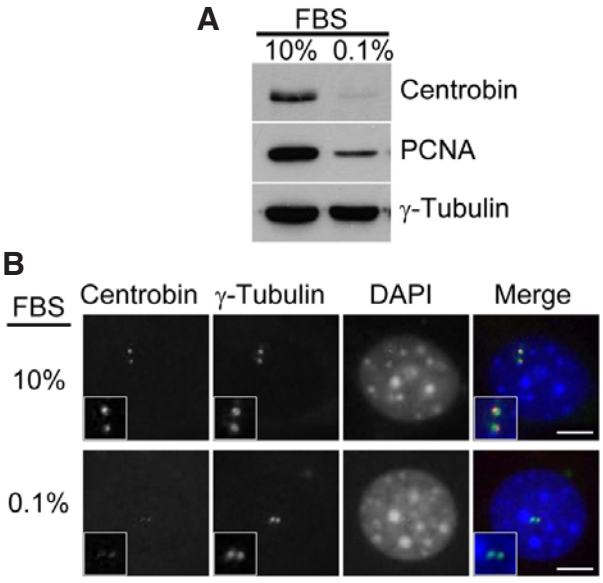

Fig. 2. Centrobin expression in the serum-starved NIH3T3 cells. The NIH3T3 cells were cultured in the medium containing 10% or 0.1% FBS for 6 d. (A) Immunoblot analysis of centrobin. The PCNA and γ-tubulin levels were determined simultaneously as controls. (B) Immunocytochemical analysis of centrobin. Representative NIH3T3 cells were immunostained with the antibodies against centrobin (red) and γ-tubulin (green). DNA was stained with DAPI (blue). Scale bars, 5 μm.

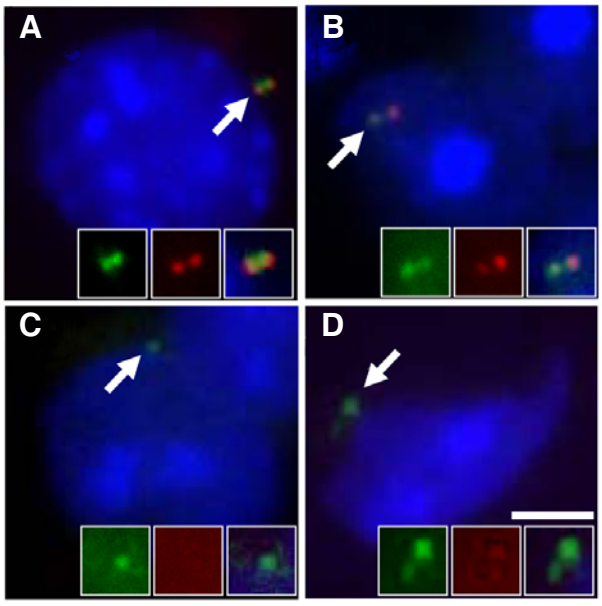

Fig. 3. Immunostaining of centrobin in the mouse testicular germ cells. Isolated mouse testicular germ cells were co-immunostained with antibodies against centrobin (red), and γ-tubulin (green). The developmental stage of the testicular germ cells was determined by morphology of the nucleus, which was stained with DAPI (blue). The centrosomes marked with arrows are magnified and viewed as γ-tubulin (green), centrobin (red) and merged. (A) Spermatocyte, (B) Early round spermatid, (C) Late round spermatid, (D) Elongated spermatid. Scale bar, 5 μm.

Immunohistochemical analysis of centrobin in the mouse testis

Subcellular localization of centrobin was determined in isolated mouse male germ cells. The results showed that centrobin was localized at the centrosomes of spermatocytes and early round spermatids (Figs. 3A and 3B). No specific signal was observed in late round spermatids and elongated spermatids (Figs. 3C and 3D). A close examination revealed that the staining patterns of centrobin and γ-tubulin did not overlap completely. Spermatocytes, which are at G2 phase of the meiotic cell cycle, had two γ-tubulin signals each of which contained a pair of the mother and daughter centrioles. The centrobin antibody immunostained only a fraction of the γ-tubulin signals in the spermatocytes (Fig. 3A). Since the round spermatid is formed right after meiosis, it contains a mother and a daughter centriole which are immunostained separately with the γ-tubulin antibody. The centrobin antibody specifically immunostained the smaller γ-tubulin dot which represents the daughter centriole (Fig. 3B). This centrobin localization pattern is consistent with the previous report in which centrobin is daughter-centriole-specific (Zou et al., 2005).

To determine the developmental stage-specific expression of centrobin during spermatogenesis, we carried out immunohistochemical analysis with mouse testis. Within a given cross section of a seminiferous tubule, there exists a characteristic association of germ cells in particular stages of spermatogenesis. In the mouse, the cycle of the seminiferous epithelium has been divided into 12 stages, each with an associated characteristic set of spermatogenic cells. The centrobin signal was detected at the centrosomes of spermatogonia and spermatocytes of all tubules (Fig. 4A). The centrobin signals at round spermatids were observed up to stages II-III but disappeared afterward. The γ-tubulin signal was detected in mouse male germ cells of all stages except elongated spermatids after stage VI (Fig. 4A). The developmental stage-specific expression of centrobin and γ-tubulin was summarized in Fig. 4B.

Centriole duplication occurred between the first and second meiotic divisions of the mouse male germ cells

In order to determine centrobin expression during meiosis, we examined the stage XI-XII tubules carefully (Fig. 5). The first and second meioses were distinguishable based on the associated cell types of the tubule and the morphology of the spermatocytes. Spindle poles of the primary spermatocyte revealed a strong γ-tubulin signal with a partly overlapped centrobin signal (Figs. 5A and 5B). This centrobin staining pattern is identical to the spindle pole of the mitotic cells (Jeong et al., 2007). Two centrobin signals appeared at the centrosomes of the interphase secondary spermatocytes right after the first meiosis (Fig. 5C). The spindle pole at the second meiosis also had a strong γ-tubulin signal with a partly overlapped centrobin signal (Fig. 5D). These results indicate that the centriole duplication should have occurred at interphase between the first and second meiotic divisions of the mouse male germ cells.

Immunohistochemical analysis of centrobin in the ovary and thymus

We examined centrobin expression in selected tissues such as ovary. Immunohistochemical analysis revealed that centrobin was located at the centrosomes of both the ovarian follicular cells and the corpus luteal cells (Fig. 6). However, the centrobin levels relative to γ-tubulin looked higher in the ovarian follicle cells than in the corpus luteal cells (Fig. 6).

The thymus is one of the tissues in which centrobin was ex-

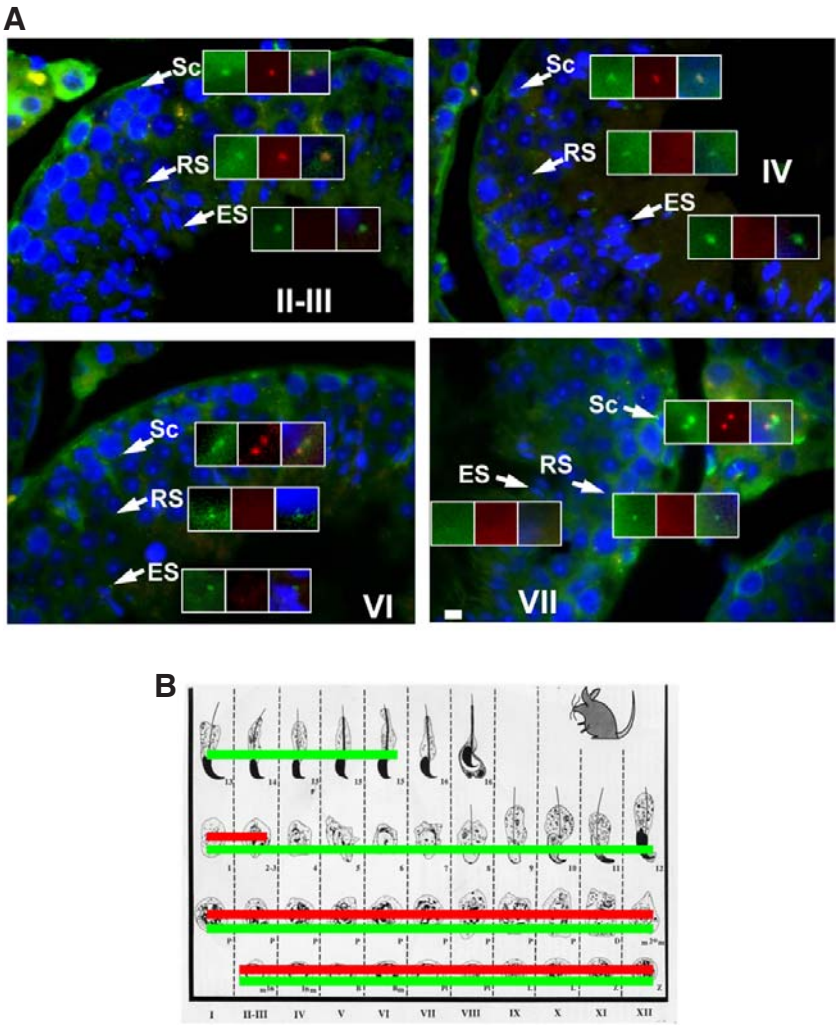

Fig. 4. Developmental stage-specific expression of centrobin in the mouse male germ cells. (A) The mouse testis sections were co-immunostained with the antibodies against γ-tubulin (green) and centrobin (red). DNA was stained with DAPI (blue). Developmental stages of the seminiferous tubules are indicated by roman numerals. Centrosomes in the spermatocytes (Sc), round spermatids (RS) and elongated spermatids (ES) are marked with arrows, and magnified in the boxes as γ-tubulin (green), centrobin (red) and merged. Scale bar, 10 μm. (B) Summary of γ-tubulin (green) and centrobin (red) expressions during development of the mouse male germ cell.

pressed abundantly (Fig. 1). The immunohistochemical analyses revealed that the centrobin protein was detected in both the cortical and the medullary cells (Fig. 7). However, intensities of the centrobin signals in the cortical cells were stronger than those in the medullary cells (Fig. 7). Intensities of the centrosomal γ-tubulin signals were more or less the same in all thymic cells, suggesting that centrobin is more abundant in the cortical cells that proliferate actively. Diffused γ-tubulin signals are staining artifact.

DISCUSSION

Immunohistochemical analysis in this study revealed that centrobin is located at the centrosomes of all mouse tissues examined except the postmeiotic spermatids of late stages. This suggests that centrobin is a core constituent of the centrosome. Absence of the centrobin at the postmeiotic spermatids may be related to the centrosome reduction phenomenon. During mouse spermiogenesis, the centrosome progressively loses its constituents and eventually disappears in mature spermaotozoa (reviewed by Manandhar et al., 2005). Our results indicated that centrobin is dissociated from the centrosome earlier than γ-tubulin.

The centrobin levels were not identical in all cell types, but they appeared to be correlated with the proliferation activity of the tissue. For example, centrobin levels were reduced in the serum-starved NIH3T3 cells at centrosomes. The centrobin levels were higher in proliferating ovarian follicular cells than in dormant corpus luteal cells. In the thymus, the centrobin levels were higher in the cortex where proliferating thymocytes were concentrated than in the medulla where post-mitotic plasma cells and macrophages accumulate. These observations are consistent with the previous reports in which centrobin was critical for centriole duplication and spindle assembly during the cell cycle (Jeong et al., 2007; Zou et al., 2005). However, a residual amount of centrobin in the resting cells may be important for maintaining integrity of the centrosome.

Centrosome duplication is tightly regulated to occur once each cell cycle in most somatic cells (reviewed by Loncarek and Khodjakov, 2009). Centrosome duplication is initiated by a number of protein kinases whose activities are induced at G1/S phase (reviewed by Nigg, 2007). The once-only control of centrosome duplication occurs at the late M phase, when the centriole becomes disengaged (Tsou and Stearns, 2006). Exceptional centrosomal behaviors are expected in the male germ cells, which must follow two consecutive meiotic divisions without nuclear replication. Observations by electron microscopy have indicated that there are at least two types of centrosomes, which behave differently during meiosis. A single centriole was found in early spermatids of some insects, proboscises, nematodes, and

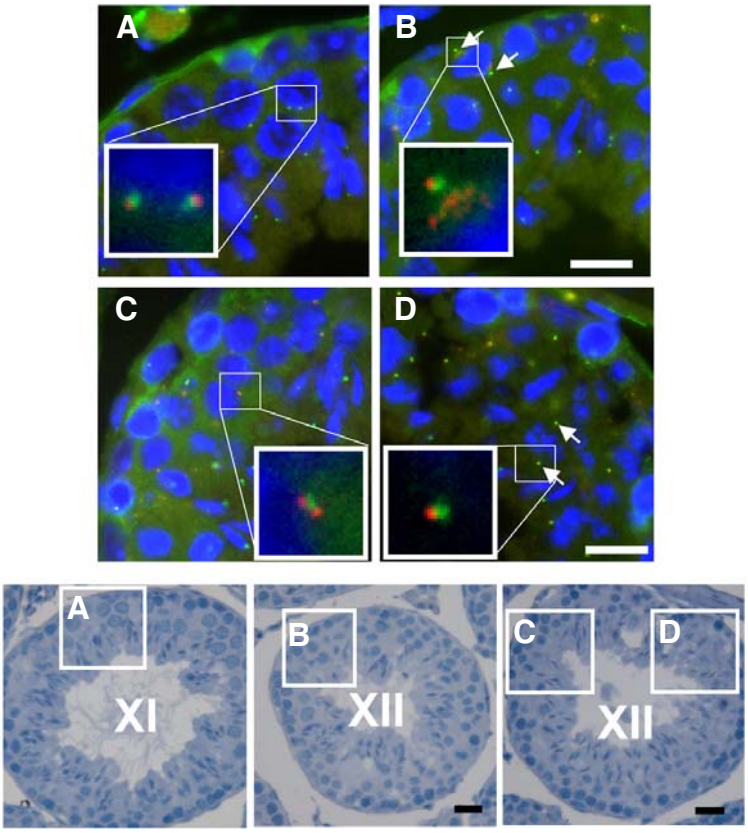

Fig. 5. Centrosome duplication during the meiotic germ cell division. The testis sections were co-immunostained with the antibodies against centrobin (red) and γ-tubulin (green). DNA was stained with DAPI (blue). Developmental stage XI and XII tubules were determined by hematoxylin staining shown on the bottom. Representative centrosomes in the meiotic germ cells are marked with the boxes and magnified. Spindle poles in the first and second meiotic metaphase cells are indicated with arrows. Scale bars, 30 μm. (A) A pair of centrosomes in the G2 primary spermatocyte, (B) A spindle pole of the first meiotic metaphase cell, (C) A duplicated centrosomes in the secondary spermatocyte before meiosis, and (D) A spindle pole of the second meiotic metaphase cell.

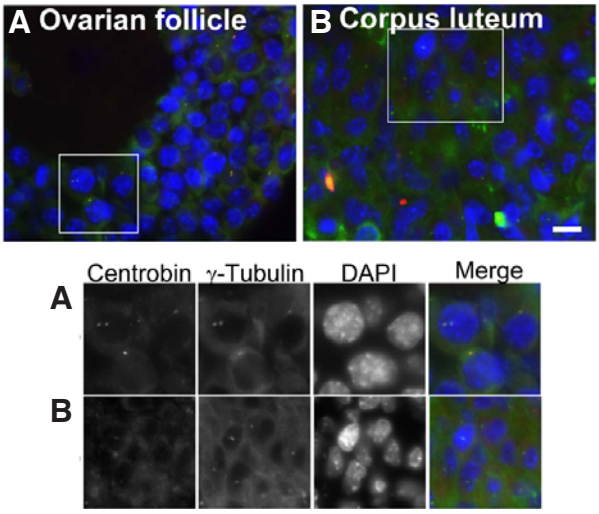

Fig. 6. Immunostaining of centrobin in the ovary. The adult ovary was co-immunostained with the antibodies against centrobin (red) and γ-tubulin (green). DNA was stained with DAPI (blue). (A) Ovarian follicle, (B) Corpus luteum. Magnified views were shown in the boxes. Scale bar, 10 μm.

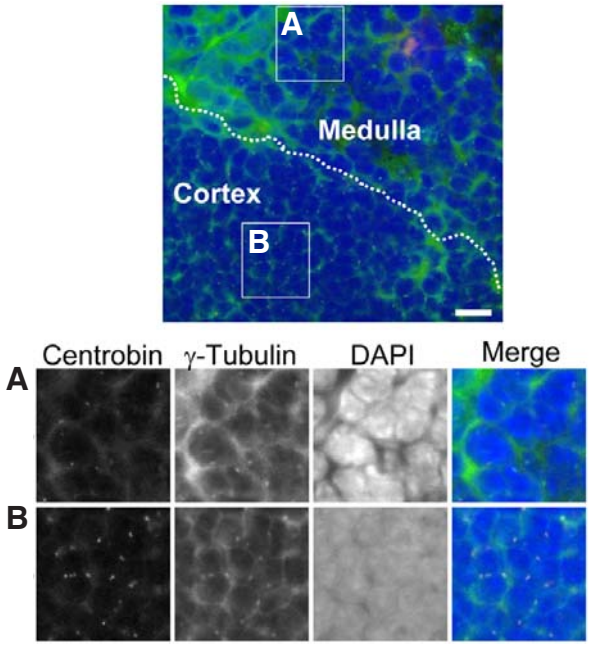

Fig. 7. Immunostaining of centrobin in the thymus. The thymus was immunostained with the antibodies against centrobin (red) and γ-tubulin (green). DNA was stained with DAPI (blue). The dotted line indicates the border between the cortex and medulla of the thymus. (A) Medullar, (B) Cortex. Magnified views of the boxes within the figure are shown on the bottom. The diffused γ-tubulin staining between cells are artifact. Scale bar, 10 μm.

earthworms, probably due to two consecutive segregations of two pairs of centrioles without duplication (reviewed by Krioutchkova and Onishchenko, 1999). In other animals, including mollusks, arthropods and vertebrates, two centrioles were detected in their mature spermatozoa (reviewed by Krioutchkova

and Onishchenko, 1999). In this case, the correlation between nuclear and centriolar cycles may be disrupted temporarily so that the centriole is duplicated during meiosis. Centrobin was known to be dissociated from the centriole that would become the mother and was detected at a nascent procentriole during centriole duplication (Jeong et al., 2007). This gave us a unique opportunity to pinpoint the exact timing of centriole duplication during meiosis. Using the centrobin antibody, we observed two daughter centrioles in the secondary spermatocyte at prophase. This is the first evidence that the centrosome duplication occurs at interphase of the second meiosis division of the mouse spermatocyte.

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