An EF-hands protein, centrin-1, is an EGTA-sensitive SUMO-interacting protein in mouse testis

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ABSTRACT

A multifunctional calcium-binding protein, centrin-1, is specifically expressed in male germ cells, certain neurons and ciliated cells. We identified centrin-1 as a protein interacting with SUMO-2/3 using yeast two-hybrid screening of a mouse testicular cDNA library. In bead halo assays, the interaction between centrin-1 and SUMO-2/3 was reduced in the presence of EGTA and facilitated by the addition of $CaCl_2$. Immunostaining of seminiferous tubules in 35-day-old mouse testes revealed that cells in the layer containing spermatogonia showed colocalization of SUMO-2/3 with centrin-1 in cytoplasmic spots. Identification of centrin-1 as the EGTA-sensitive SUMO-2/3-interacting protein indicates the possible role of calcium in modulating the centrin-1–SUMO-2/3 interaction and suggests the importance of this interaction in mouse testis.

INTRODUCTION

Spermatogenesis is essential for sexual reproduction in animals. It is triggered by sequential mitotic divisions of spermatogonia in the testis, followed by their differentiation into spermatocytes. After two meiotic divisions, spermatocytes develop into haploid spermatids possessing half the normal complement of genetic material and then into spermatozoa, the mature male gametes in many sexually reproducing organisms. This complex process appears to be controlled by cooperation between several hormones and testicular somatic cells that leads to subsequent changes in a wide variety of cell morphology and cellular signaling regulated, in part, by post-translational protein modifications.[1]

The small ubiquitin-related modifier (SUMO) is a post-translational protein modifier, similar to ubiquitin in terms of its structure and its mechanism of conjugation to target proteins. SUMO is involved in many diverse cellular functions, including DNA replication, repair and recombination, RNA transcription, nucleocytoplasmic trafficking, and protein stability.[2,3] In higher eukaryotic cells, such as in the human and mouse, at least two paralogs, SUMO-1 (SMT3C) and SUMO-2/3 (SMT3A/B), have been reported.[4] Although several groups have tried to elucidate the role of the SUMO modification pathways in the differentiation of male germ cells and to seek key signaling molecule(s) that might link the SUMO pathways with the onset and/or progression of meiosis during spermatogenesis, most of them remain largely uncharacterized.[5–7]

Centrins are relatively small acidic proteins that contain four helix–loop–helix motifs, the so-called EF-hands, which represent potential calcium binding sites.[8–10] In mammals, at least three centrin proteins have been identified so far,[10-13] centrin-1 and centrin-2 share approximately 80–90% identity in vertebrates, and sequences of the yeast centrin and the related mammalian centrin-3 isoform have amino acid identities of only about 55%, compared with the other isoforms. Human centrin-1 and centrin-2 have been reported to bind preferentially to SUMO-2/3, unlike SUMO-1.[14] Nucleocytoplasmic shuttling of centrin-2 is controlled by the SUMO modification system and the xeroderma pigmentosum group C (XPC) protein, suggesting the importance of a dynamic interplay among centrin-2, SUMO-2/3 and nuclear/chromatin protein(s). In line with studies on the subcellular localization of centrin-2 and its

associated factors, it is interesting to note that centrin-2 might function in mRNA export and this function seems to be conserved.[15,16] Thus, it is reasonable to speculate that centrin's subcellular localization can influence its associated molecules/factors in the nucleus. In contrast to centrin-2, little is known concerning centrin-1. In part, this is because of its restricted expression in the testis and retina, which have flagella and cilia, respectively,[11–13] whereas centrin-2 is expressed in most cell types except for the testis.

Here we describe the isolation of centrin-1 cDNA in the yeast two-hybrid screening of a mouse testicular cDNA library using the GAL4–SUMO-3–SUMO-3 fusion protein as a bait. This confirmed previous data indicating that centrin-1 and centrin-2 bind SUMO-2/3 in the yeast two-hybrid system.[14] In addition, we show here for the first time that the *in vitro* interaction of centrin-1 with SUMO-3 is reduced in the presence of EGTA and influenced by calcium, suggesting a possible role of metal ions ~~flux~~ in controlling the centrin-1–SUMO-2/3 interaction. Moreover, biochemical and immunostaining experiments using the adult mouse testis suggested that there is spatiotemporal interaction of the two proteins in the cells localized at the layer containing spermatogonia in seminiferous tubules. Therefore, we propose centrin-1 as a previously unappreciated form of ~~calcium-modulated~~ EGTA-sensitive SUMO-interacting protein that might play a role in the differentiation of male germ cells.

MATERIALS AND METHODS

Plasmids

To generate enhanced green fluorescent protein (EGFP)–centrin-1 and glutathione S-transferase (GST)–centrin-1 expression vectors, mouse centrin-1 was amplified by polymerase chain reaction (PCR) and cloned into the pEGFP-C2 (Clontech) and pGEX (GE Healthcare) vectors, respectively. Centrin-2, SUMO-1/2/3 and MCAF1-SIM expression plasmids were as described previously.[17,18,19]

Antibodies

A rabbit polyclonal antibody against SUMO-2/3 was used as described.[17,18] Anti-centrin-1 and anti-γ-tubulin antibodies were obtained from Santa Cruz Biotechnology and Sigma-Aldrich, respectively.

The secondary antibodies used in this study were as described.[17,18]

Bead halo assay

This method was based on a modification of a low-affinity protein interaction procedure, known as bead halo assay, described previously.[20] In brief, a 50 ml culture of *Escherichia coli* carrying pET28-EGFP-SUMO-3 was used to express recombinant SUMO-3 fused to EGFP. The bacterial pellet was dissolved in 5 ml of phosphate-buffered saline (PBS, pH 7.0), followed by sonication. The sonicated bacterial lysate was centrifuged and the supernatant was stored at –20^{o}C until use (approximately 0.01 mg EGFP-SUMO-3/ml of bacterial lysate). In the experiments described in Figs 2C and 2D, expressed EGFP-SUMO-3 was purified using Ni-beads according to the manufacture's protocol and added to the reaction mixture in the presence or absence of appropriate amount of $CaCl_2$ as indicated in the figures. The GST-centrin-1 and -centrin-2 were expressed in *E. coli* and were then incubated with glutathione-Sepharose 4B beads (GE Healthcare). We used 10 µl of packed beads for bacterial lysate prepared from 10 ml of bacterial culture. GST-fusion proteins were immobilized at high concentrations on the surface of the Sepharose beads. Approximately 5-10 µg of GST-fusion proteins were bound to 10 µl of packed beads. To elucidate the effect of calcium described in Figs 2C and 2D, the beads were prepared with PBS containing 2 mM EGTA followed by extensive washing with PBS without EGTA. The beads were resuspended as a 50% slurry in PBS and a 2 µl portion of the slurry, in which approximately 30 particles of beads were included, was mixed, on a 76 × 26 mm Micro Slide Glass with 2.0 µl of either bacterial lysate or purified EGFP-SUMO-3 in the presence or absence of EGTA/$CaCl_2$. As soon as the incubation started, the beads were viewed in real-time under equilibrium conditions and photographed using a BIOREVO BZ-9000 fluorescence microscope (Keyence). When EGFP-SUMO-3 binds to an immobilized GST-centrin protein, the interaction is visible through GFP filters as a halo of fluorescence around the beads.

Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mixture

(Sigma-Aldrich) with 5% fetal calf serum and antibiotics, in 5% CO_2. Transfection was carried out as described.[17] Indirect immunofluorescence was carried out as described.[18]

Immunoblotting and immunoprecipitation analyses

Testes (0.01–0.07 g) obtained from different ages of mice were homogenized in 3xSDS-sample buffer with a sonicator (Tomy-UD201). The supernatant (2 mg/ml protein) was used in immunoblotting and immunoprecipitation analyses.[18]

Immunohistology

C57BL/6 mice (purchased from Kyudo Co., Ltd., Japan) were used and were maintained, operated on, euthanized and dissected according to the guidelines of Kumamoto University. A small piece of testicular tissue was subjected to 6 μm cryosections (MICROM HM505N, Uniscience). The sections were fixed by immersion in 4% paraformaldehyde in PBS and were immunostained using anti-centrin-1 (dilution 1/200) and anti-SUMO-2/3 antibody (dilution 1/100). Nonspecific antibody-binding sites were blocked using Roche blocking reagent (Roche). To visualize the nucleus, the sections were incubated in DAPI for 10 min. The sections were observed using BIOREVO BZ-9000.

RESULTS

Identification of centrin-1 as a protein interacting with SUMO-2/3 and characterization of its subcellular localization in cultured cells

To identify a protein(s) that interacts with SUMO, we performed a GAL4-based yeast two-hybrid screening using the SUMO-3–SUMO-3 fusion protein as bait.[17,18] One of the interesting clones encoded a mouse homologue of centrin-1, a relatively small acidic protein that is expressed specifically in the testis and retina and contains EF-hands, which represent potential calcium-binding sites. As shown in Figs 1A and 1B, a bead halo assay—a simple and rapid procedure that was developed to detect low affinity interactions in real time under equilibrium binding conditions demonstrated that centrin-1 interacts

directly with SUMO *in vitro* in preference to SUMO-3, consistent with a previous observation.[14]

Despite the known localization of centrin-2 at the centrosome as well as in the nucleus and at the nuclear rim of mammalian cultured cells, the subcellular localization of centrin-1 in mammalian cultured cells remained unclear.[14,15,21] Therefore, to clarify its subcellular localization and to test on any functional overlap between centrin-1 and centrin-2 at the centrosome, we transiently expressed EGFP–centrin-1 in HeLa cells. As shown in Fig. 1C, green fluorescence was detected in both the cytoplasm and the nucleoplasm, with prominent accumulation as spots in the cytoplasm ~~and the nuclear rim~~. Because the cytoplasmic spots were co-stained with anti-γ-tubulin antibody, this transient expression of EGFP-centrin-1 revealed that centrin-1 can be targeted to the centrosome in Hela cells. It should be noted that similar patterns were observed when EGFP-centrin-1 was expressed in a mouse germ seminoma cell line, GC1 (data not shown), suggesting that centrin-1 accumulates in both the nucleoplasm and cytoplasm with a potential to localize to the centrosome in mammalian cells. Taken together, these data imply that centrin-1 is a protein that interacts with SUMO-2/3 and has ability to localize at multiple subcellular compartments, including the centrosome.

The interaction between centrin-1 and SUMO-3 is modulated by calcium

Given that the major domains of centrin-1 comprise calcium-binding EF-hand motifs,[8-10] we investigated whether the affinity of centrin-1 to SUMO-2/3 might be affected by calcium. To do this, we first wished to determine whether centrin-1 binding to SUMO-3 could be reduced by the addition of EGTA, a calcium chelating agent, in the bead halo assay. In Figs 2A and 2B, we prepared the beads and incubated them with the lysate in a buffer containing 0 mM or 2 mM EGTA, respectively. Compared with the incubation conditions of 0 mM EGTA, addition of EGTA produced significant reduction of green fluorescence around the centrin-1 beads. In contrast, EGTA had little effect on the green fluorescent signals around the beads containing GST-MCAF1-SIM. Given that *in vitro* binding and nuclear magnetic resonance (NMR) spectroscopy analyses concerning the SUMO-2/3–MCAF1-SIM interaction revealed that there is no requirement of calcium and/or other metal ions for interaction, we chosen MCAF1-SIM as a suitable control protein that binds to SUMO in a manner independent of EGTA.[17,22] Thus our data

indicate that EGTA effectively reduces the association of centrin-1 with SUMO-3, and that the effect of EGTA was specific to the interaction between centrin-1 and SUMO-3. Moreover, a bead halo assay revealed that GST-centrin-2, another centrin family protein which has been reported to bind SUMO-2/3,[21] reduced its ability to bind with EGFP-SUMO-3 in the presence of EGTA (Fig. 2E), implying that the EGTA-sensitive binding to SUMO-2/3 might be a common feature among centrin-1 and -2 family proteins.

Given that EGTA is known as a calcium chelating agent, we then tested whether an increased concentration of calcium in the reaction buffer would facilitate the binding of SUMO-3 to centrin-1. When conducting this experiment, we expected that centrin-1 prepared by treatment with EGTA might be a calcium-free form that would incorporate calcium rapidly when $CaCl_2$ was added to the reaction buffer (see Materials and Methods section in detail). As shown in Figs 2C and 2D, green fluorescence was not detected when the bead halo assay was performed in the absence of $CaCl_2$. In contrast, green fluorescence appeared around the beads when the assay was carried out in the presence of 0.1 mM, 1 mM or 2 mM $CaCl_2$. Since no significant effect of addition of $CaCl_2$ on the GST–MCAF1-SIM interaction (Figs 2C and D), our results suggest that the interaction between centrin-1 and SUMO-2/3 can be facilitated by calcium.

Mapping of SUMO-interacting domain/motif in centrin-1

Next, to determine the region responsible for binding to SUMO-2/3, we generated a series of deletion mutants of recombinant centrin-1 and performed a bead halo assay. Because many SUMO-binding proteins reported so far contain a short polypeptide sequence which has key hydrophobic residues often flanked by acidic residues, referred to as SUMO interacting motif (SIM),[17,18,22-26] we expected that centrin-1 was a canonical SIM-containing protein. As shown in Figs 3A and 4B, GST protein fused to a C-terminal fragment of centrin-1, residues 94–172 bound strongly with EGFP-SUMO-3; conversely the binding of GST proteins fused to the N-terminal fragment 1–98 or to each of the EF-domain (residues 1-64 for EF-1, residues 65-98 for EF-2, residues 94-145 for EF-3 and residues 139-145 for EF-4, respectively) was notably weaker than that for the residues 94–172. These results indicated that the region

between amino acids 94-172 of centrin-1, which includes both EF-3 and EF-4 domains, is important for efficient binding to SUMO-3, although other region of centrin-1, including EF-1 and EF-2, might also contribute to the interaction with SUMO-3 to some extent.

To further localize SIM in the region between amino acids 94-172 of centrin-1, we looked for the canonical SIM in amino acids 94-172. Although a sequence perfectly matched to the canonical SIM was not detected in this region, we found that the amino acids 133-142 (LGESLTDEEL) were reasonably similar to the canonical SIM. To elucidate whether this polypetide functions in SUMO binding, we made a GST protein fused to amino acids 133–142 (GST-133-142) and performed a bead halo assay. As shown in Figs 3A and 3B, GST-133-142 fusion protein failed to bind EGFP-SUMO-3, suggesting that, although the sequence showed similarity to the canonical SIM, this polypeptide was not sufficient for SUMO-binding. Thus we failed to map a SUMO interacting motif to a small region in centrin-1, suggesting that centrin-1 contains an unusual SIM or binds to SUMO in a manner different from the interaction with the canonical SIM.

Immunohistology of the centrin-1–SUMO-2/3 interaction in mouse seminiferous tubules

Because centrin-1 is expressed exclusively in male germ cells, certain neurons and ciliated cells, we chose the mouse testis to investigate the possible *in vivo* interaction between centrin-1 and SUMO-2/3.[11–13] For this, we first determined the expressions of centrin-1 and SUMO-2/3 at the protein level. We performed immunoblot analyses using lysates from mice testes and detected centrin-1 in 21-, 28- and 30-day old mouse testes (Fig. 4A). In these extract, SUMO-2/3 was also detected, suggesting that both centrin-1 and SUMO-2/3 were expressed in adult mouse testes.

Next, to investigate whether centrin-1 and SUMO-2/3 interacted *in vivo* and to elucidate any physiological significance, we performed immunostaining on the seminiferous tubules to test whether we could visualize a subset of testicular cells that showed colocalization of these proteins. Sections of individual tubules obtained from the adult mouse testis contain the full range of spermatogenic stages, which can be divided spatially into three cell layers. The outermost layer of each tubule contains spermatogonia and interspersed Sertoli cells; the middle zone contains primary and secondary

spermatocytes and the innermost zone contains spermatids and spermatozoa. Thus, immunostaining of the adult seminiferous tubules allowed investigation of the centrin-1–SUMO-2/3-interaction among multiple cell types during the course of spermatogenesis. No signal was observed in control experiments, either omitting the incubation with anti-centrin-1 or anti-SUMO-2/3 antibodies (data not shown). However, as shown in Fig. 4B, a section of individual tubules obtained from a 35-day-old mouse testis showed positive signals, suggesting that the immunofluorescent signals in the sections were specific to the antibodies.

When the sections were observed, it was obvious that the cells present in the middle zone enriched with primary and secondary spermatocytes (region #3 in Fig. 4B) showed significant labeling with the anti-SUMO-2/3 antibody. Because previous reports demonstrated that the components in the SUMO pathway, including SUMO-2/3, SUMO-1, Ubc9 (SUMO-E2), Zimp7 (PIAS family of SUMO-E3) and PML (SUMO-substrate), were localized to the XY body in pachytene and diplotene spermatocytes, we speculate that the prominent signals detected by anti-SUMO-2/3 antibody in our specimen represent the XY-body.[5-7,27] In contrast, centrin-1 signals at the XY-body were barely detected, indicating that centrin-1 might not be a major component of the XY body in spermatocytes. On the other hand, the cytoplasmic spots detected by the anti-centrin-1 antibody were apparent in many cells not only in the mid zone but also the outer (region #1 and #2) and inner (region #4) layers, suggesting that centrin-1 is a component of the centrosome during spermatogenesis, consistent with a previous observation.[28] Intriguingly, when observing the immunostaining pattern of anti-centrin-1 antibody in consecutive sections, there were multiple cells containing cytoplasmic spots that costained with anti-centrin-1 and anti-SUMO-2/3 antibodies in cells in the outer zone, but not in the middle and inner layers. Thus these observations suggest that there might be a temporal difference in antigen availability of SUMO-2/3 in the cytoplasm, possibly at the centrosome region. Alternatively, the localization of SUMO-2/3 in the cytoplasm, in particular at around the centrosomal region, might be regulated temporally during spermatogenesis. Taken together, these immunostaining results on the mouse testis suggest that there is interaction between centrin-1 and SUMO-2/3 in the cytoplasmic spots in cells present in the outer zone of seminiferous tubules, containing mostly spermatogonia.

DISCUSSION

Our studies revealed that centrin-1 preferentially binds to SUMO-2/3 rather than SUMO-1 (Fig.1), and that their interaction appears to be reduced in the presence of EGTA (Figs 2A and 2B). The EGTA-sensitive SUMO-binding was also observed when centrin-2, another centrin family protein in mammals, was subjected to a bead halo assay, suggesting that EGTA-sensitive SUMO-binding property might be conserved among centrin-1 and -2 family proteins (Fig. 2E).

Protein structures at high resolution based on X-ray crystallographic data have been determined for yeast and human centrin.[29,30] The molecular structure of centrin includes four EF-hands, which comprise the prototypical bivalent metal ion binding helix–loop–helix motif. *Chlamydomonas* centrin binds four calcium ions with dissociation constants ranging from 1 nM to 0.1 mM, while only the carboxy-terminal EF-hands of human centrin-2 bind calcium ion at physiological concentrations.[30–33] Therefore it is reasonable to speculate that depletion and addition of calcium may influence centrin-1-SUMO-2/3 interaction. Indeed the addition of EGTA, which may cause the reduction of calcium ion in the reaction buffer via its preferential chelating activity toward calcium, resulted in reduction of centrin-1's binding to SUMO-3 (Figs 2A and 2B). Furthermore, as shown in Figs 2C and 2D, we detected that nearly full recovery of EGFP-SUMO-3 binding by addition of $CaCl_2$ to the reaction buffer containing nearly negligible calcium ion. As such, these results encourage us to propose calcium is an important metal ion to regulate the interaction between centrin-1 and SUMO-2/3. However, it remains to be elucidated in detail which metal ion(s) besides calcium binds most efficiently to the EF-hands in centrin-1 or centrin-2 in our experimental conditions. We cannot rule out the possibility of substitution of other metal(s) for calcium in the context of regulating the centrin-1-SUMO interaction *in vitro* as well as *in vivo*.

Attempts to map the SUMO interacting motif (SIM) in centrin-1 led to the realization of its difficulty (Fig. 3). One feasible explanation for our failure to map a SIM to a small region in centrin-1 is that the interaction of centrin-1 to SUMO-2/3 might be distinct from the interaction of the canonical SIMs Although the structural basis of EGTA-sensitive centrin-1–SUMO-2/3 interaction should be elucidated, our *in vitro* data clearly indicate that the calcium-binding form of centrin-1 shows much higher affinity to

SUMO-2/3 than does the calcium-free form, suggesting that a metal-dependent conformational change is important for binding with SUMO-2/3. It would be intriguing to speculate that the interaction might be important for the conformational change if centrin-1 is modified by SUMO-2/3.

Immunostaining revealed colocalization of centrin-1 and SUMO-2/3 in the area containing a significant population of spermatogonia (Fig.4). We believe that this is the first approach for characterizing centrin-1–SUMO-2/3 interaction in the testis. Centrin-1 and SUMO-2/3 colocalized at cytoplasmic spots in cells present in the outer zone of the seminiferous tubule, containing mostly spermatogonia. Based on the nature of centrin-1 as a centrosomal component, this suggests that the cytoplasmic interaction between centrin-1 and SUMO-2/3 might represent a spatiotemporal regulation of centrosomal proteins during spermatogenesis. Klein and Nigg reported that human centrin-2 could be modified by either SUMO-1 or SUMO-2/3, but indicated that localization of centrin-2 to the centrosome was independent of its SUMOylation.[14] Although their results argue that centrin-1 itself could be an efficient SUMOylation substrate in the testis, we could not detect a SUMOylated form of endogenous centrin-1 using the lysates of 35-day-old mice testes, suggesting that most of the endogenous centrin-1 in testis was modified very poorly by SUMO (if at all), or that any SUMOylated centrin-1 in the testis was unstable (unpublished data). Thus, we assume that colocalization of centrin-1 and SUMO-2/3 at the cytoplasmic spots might represent an interaction of centrin-1 with SUMOylated proteins that is distinct from SUMOylated centrin-1. In future experiments, proteomic analysis of SUMOylated proteins in the mouse testis should be explored to identify a centrosomal protein(s) that can be SUMOylated and interact with centrin-1.

In conclusion, the testis-specific and conspicuous spatiotemporal expression, together with the calcium-binding property of centrin-1, suggest that this EGTA-sensitive interaction with SUMO-2/3 might be very important for the differentiation of male germ cells. Although further studies will be required to provide insights into the physiological role of the interaction, this study improves understanding of how protein SUMOylation is involved in centrin-1/2- or centrosome-function during spermatogenesis as well as in the context of calcium signaling.

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FIGURE LEGENDS

Figure 1. Centrin-1 is an EF-hands protein localized at the centrosome.

A: Preferential binding of centrin-1 with SUMO-3 in the bead halo assay. A bacterial lysate containing 0.01 mg/ml of EGFP–SUMO-1 (left) or EGFP–SUMO-3 (right) was mixed with the bead-immobilized GST–centrin-1 (upper) and GST alone (bottom), respectively. Aliquots of the recombinant EGFP–SUMO-1, EGFP–SUMO-3, GST–centrin-1 or GST alone were separated by SDS–PAGE and stained with Coomassie blue. The mixtures were treated as described in the Materials and Methods and imaged using a fluorescence microscope with an appropriate green fluorescence filter.

B: The amount of green fluorescence on the GST-centrin-1-beads was measured (n = 50 beads). a.u., arbitrary fluorescence unit.

C: Subcellular localization of EGFP-tagged centrin-1. HeLa cells were transfected with an expression vector for EGFP–centrin-1. After 24 h, the cells were fixed and green fluorescence was detected by fluorescence microscopy. To visualize the centrosome, indirect immunofluorescence was also performed using an anti-γ-tubulin antibody. Enlarged image of the boxed region is shown (inset). Merged images are on the right (Bar = 50 μm).

Figure 2. *In vitro* interaction of centrin-1 with SUMO-2/3 is facilitated by Ca^{2+}.

A: Interaction of centrin-1 with SUMO-3 was reduced in the presence of EGTA. Beads immobilized to GST–centrin-1 (upper) or GST–MCAF1-SIM (lower) were prepared and treated with a bacterial lysate containing 0.01 mg/ml of EGFP–SUMO-3 containing 0 mM or 2 mM EGTA, followed by washing the beads with PBS. Aliquots of the recombinant EGFP–SUMO-3, GST–centrin-1 or GST-MCAF1-SIM were resolved by SDS–PAGE and stained with Coomassie blue.

B: Quantification of centrin-1–SUMO-3 interaction observed in (A). The amounts of green fluorescence on the beads bound to GST–centrin-1 (left axis) or GST–MCAF1-SIM (right axis) were measured (n = 60 beads). a.u., arbitrary fluorescence unit.

C: Interaction of centrin-1 with SUMO-3 was enhanced by the addition of $CaCl_2$. The GST-centrin-1 or

GST-MCAF1-SIM expressed in *E. coli* was incubated with glutathione-Sepharose beads in PBS containing 2 mM EGTA, expecting the production of the beads associated with calcium-free form of GST-centrin-1 and –MCAF1-SIM. .Approximately 0.01 mg/ml purified EGFP-SUMO-3 in PBS containing 0.1% Triton-X100 was mixed with the bead-immobilized GST–centrin-1 (upper) or GST–MCAF1-SIM (lower) followed by incubation in buffer containing 0, 0.1, 1 or 2 mM $CaCl_2$. Aliquots of the recombinant EGFP–SUMO-3, GST–centrin-1 or GST–MCAF1-SIM were resolved by SDS–PAGE and stained with Coomassie blue.

D: Quantification of centrin-1–SUMO-3 interaction in (C). The amounts of green fluorescence on the beads bound to GST–centrin-1 (left axis) or GST–MCAF1-SIM (right axis) were measured (n = 60 beads). a.u., arbitrary fluorescence unit.

E: Centrin-2 is an EGTA-sensitive SUMO-interacting protein. Beads immobilized to GST–centrin-2 was prepared and treated with a bacterial lysate containing 0.01 mg/ml of EGFP–SUMO-3 in the presence of 0 mM (left) or 2 mM EGTA (right), followed by washing the beads with PBS. Aliquots of the recombinant EGFP–SUMO-3 or GST-centrin-2 were resolved by SDS–PAGE and stained with Coomassie blue. The amounts of green fluorescence on the beads bound to GST–centrin-2 were measured (n = 60 beads). a.u., arbitrary fluorescence unit.

Figure 3. The interaction of each centrin-1-deletion mutant with SUMO-3 was detected by the bead halo assay.

A: Schematic representation of centrin-1 and four individual EF-hands (EF-1, -2, -3, and 4), and a summary of the bead halo assay. Centrin-1 deletion constructs are represented as thin black lines. Numbers indicated on the sides of the lines correspond to amino acid residues in centrin-1. The Table on the right summarizes the interactions of each deletion mutant with EGFP-SUMO-3 in the bead halo assay shown in B. The symbols +++ represents strong binding; +, sufficient binding; -/+, weak binding; and -, negligible binding.

B: Interaction of each GST–centrin-1-deletion mutant with SUMO-3. Beads immobilized to each GST–centrin-1-deletion mutant as indicated were prepared and treated with a bacterial lysate containing

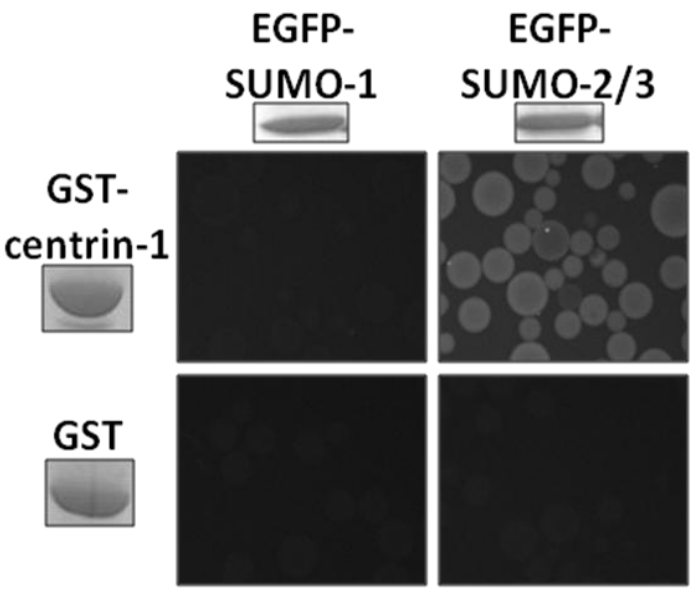
0.01 mg/ml of EGFP–SUMO-3 in PBS containing 1 mM $CaCl_2$, followed by washing the beads with PBS. Aliquots of the recombinant EGFP–SUMO-3 or GST–centrin-1 mutants were resolved by SDS–PAGE and stained with Coomassie blue.

Figure 4. Centrin-1–SUMO-2/3 interaction is detectable in the adult mouse testis.

A: Expression of centrin-1 protein in mouse testis. Testicular lysates from 21-, 28- and 30-day-old mice were used for immunoblotting analysis with anti-centrin-1 (upper) and anti-SUMO-2/3 (middle) antibodies. The Coomassie blue stained SDS–PAGE result is shown at the bottom.

B: Immunofluorescence detection of centrin-1 and SUMO-2/3 in sections of seminiferous tubules. Seminiferous tubules of 35-day-old mouse testis were sectioned and stained with DAPI, anti-centrin-1 and anti-SUMO-2/3 antibodies. DAPI stains DNA and visualizes the nucleus. The area circled by a white dotted line indicates an individual tubule. Sections of individual tubules obtained from the adult mouse testis contain the full range of spermatogenic stages, which can be divided into three cell layers. The outermost layer of each tubule contains spermatogonia and interspersed Sertoli cells (box #1 and #2); the middle zone contains primary and secondary spermatocytes (box #3) and the innermost zone contains spermatids and spermatozoa (box #4). The left panel shows the merged image of triple-stained immunofluorescence of a section. The right panel shows immunofluorescence images of each boxed region stained with anti-SUMO-2/3 (left) and anti-centrin-1 (middle) antibodies. DAPI-stained and merged images are on the right (Bar = 50 μm). The arrowheads indicate the positions of cytoplasmic spots detected by the anti-centrin-antibody. These cytoplasmic spots might represent the centrosome (and see Fig. 1C).[28]

A



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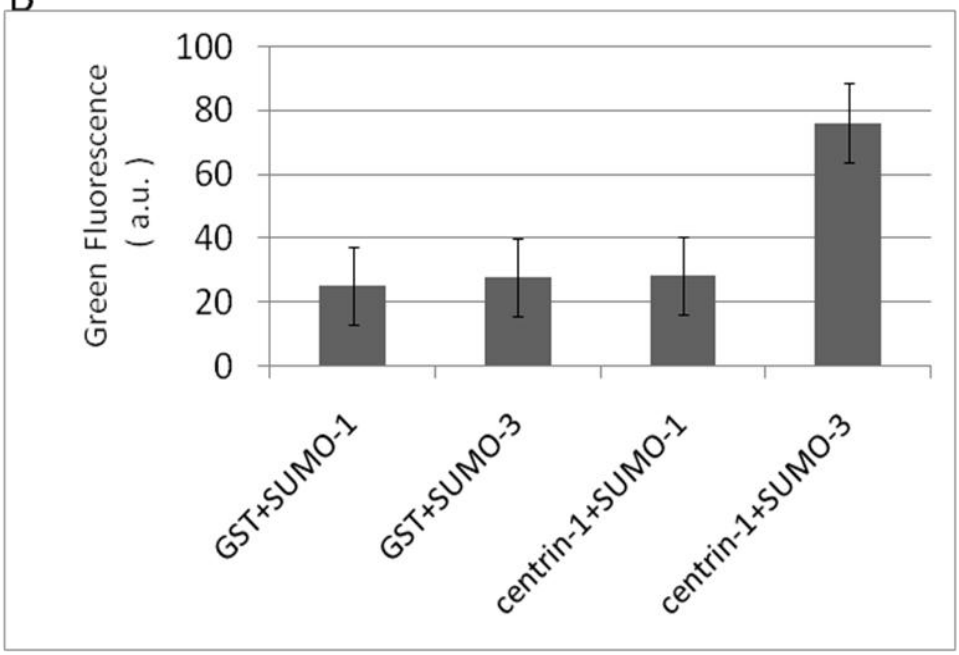
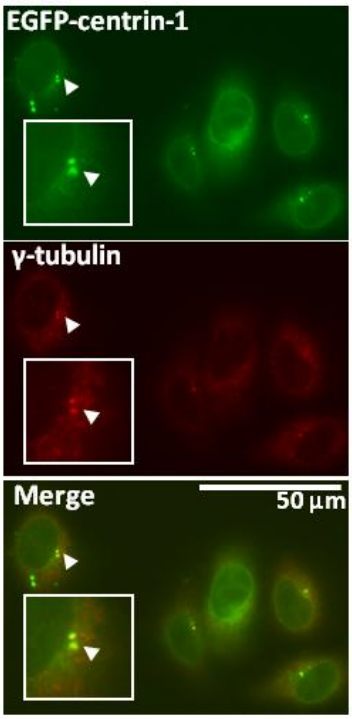


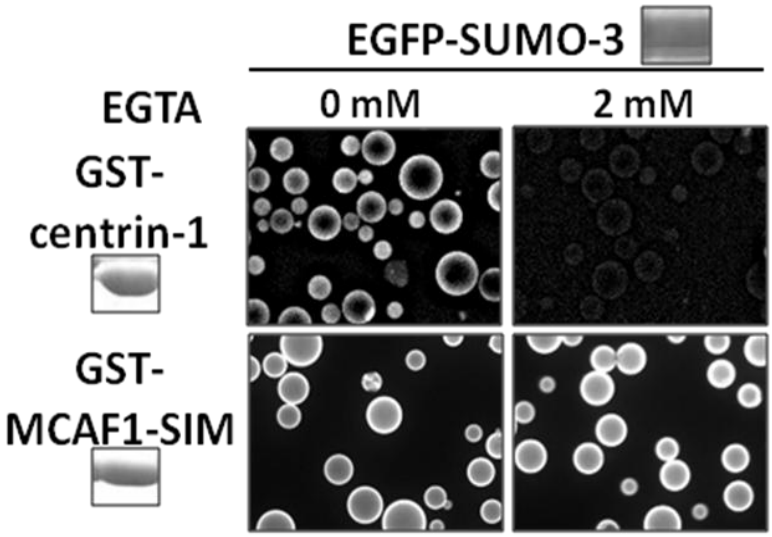
Tanaka et al. Fig. 1

C



Tanaka et al. Fig. 1

A



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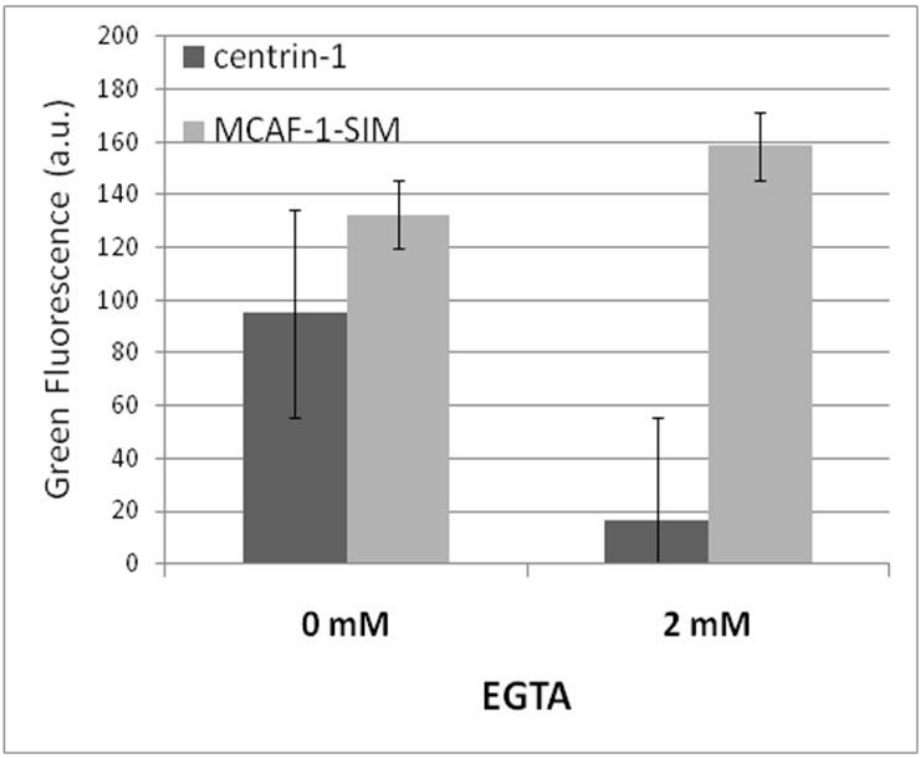
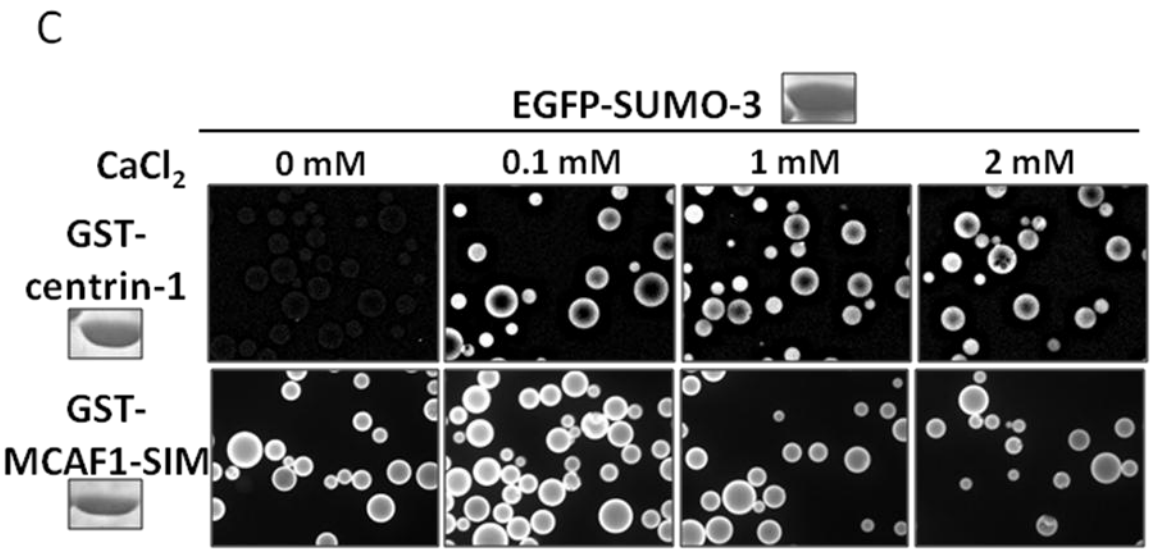
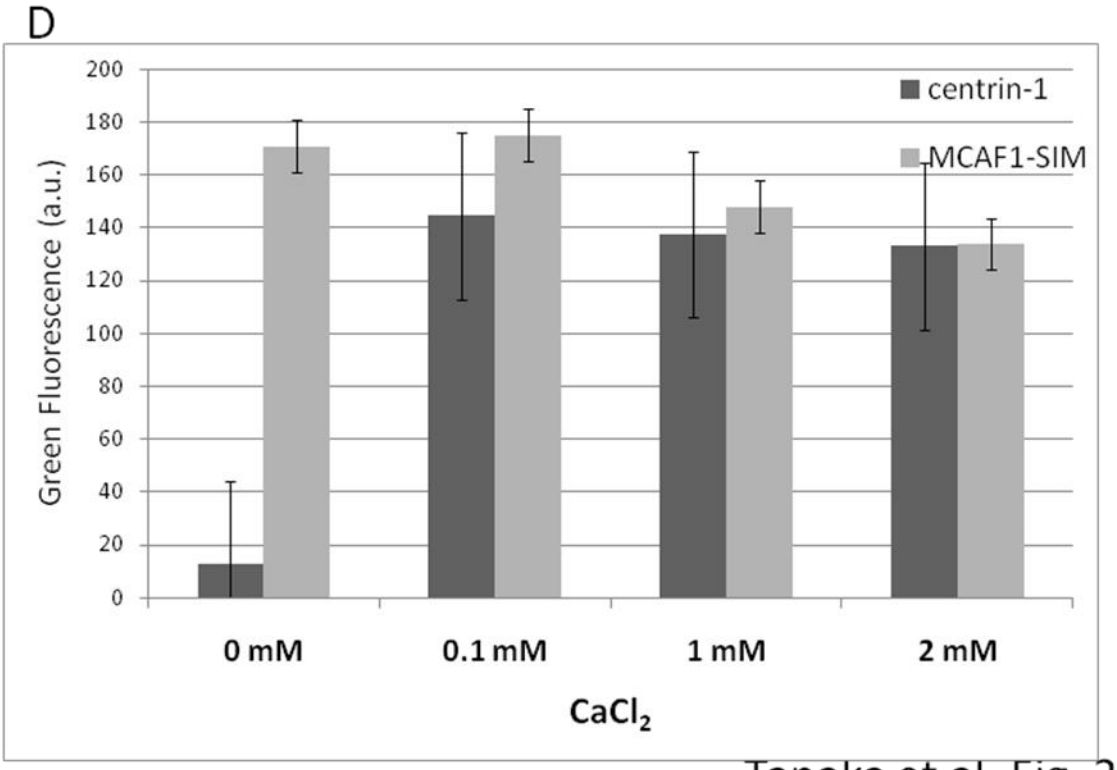


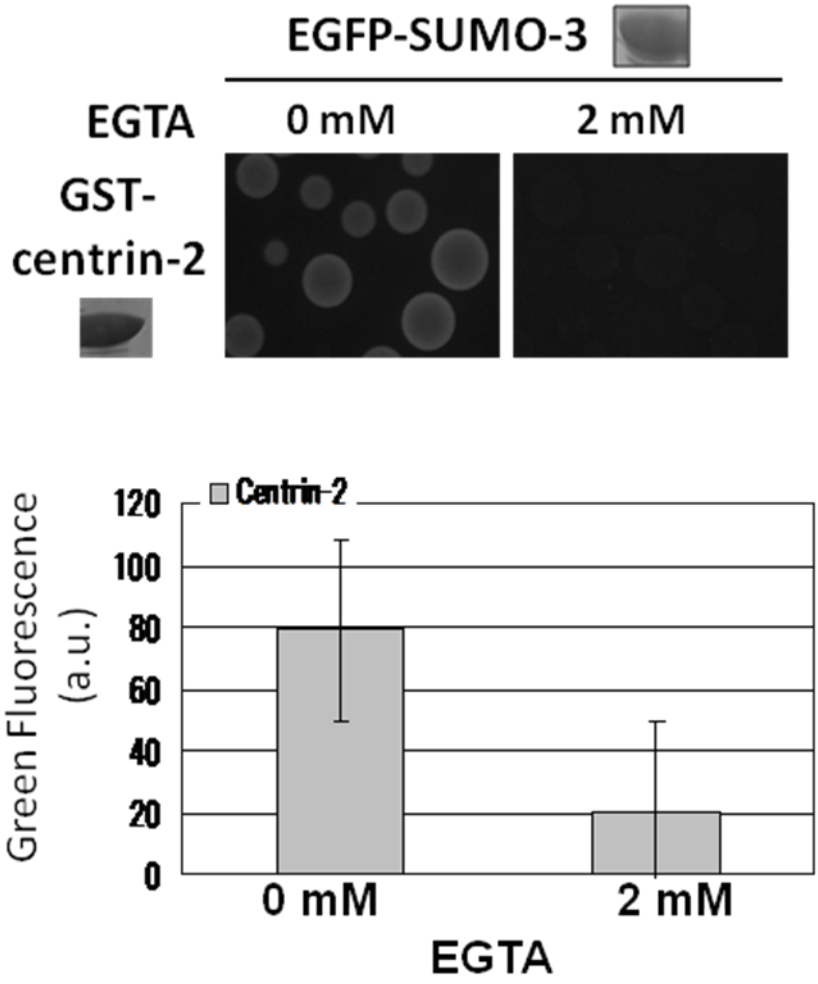
Tanaka et al. Fig. 2



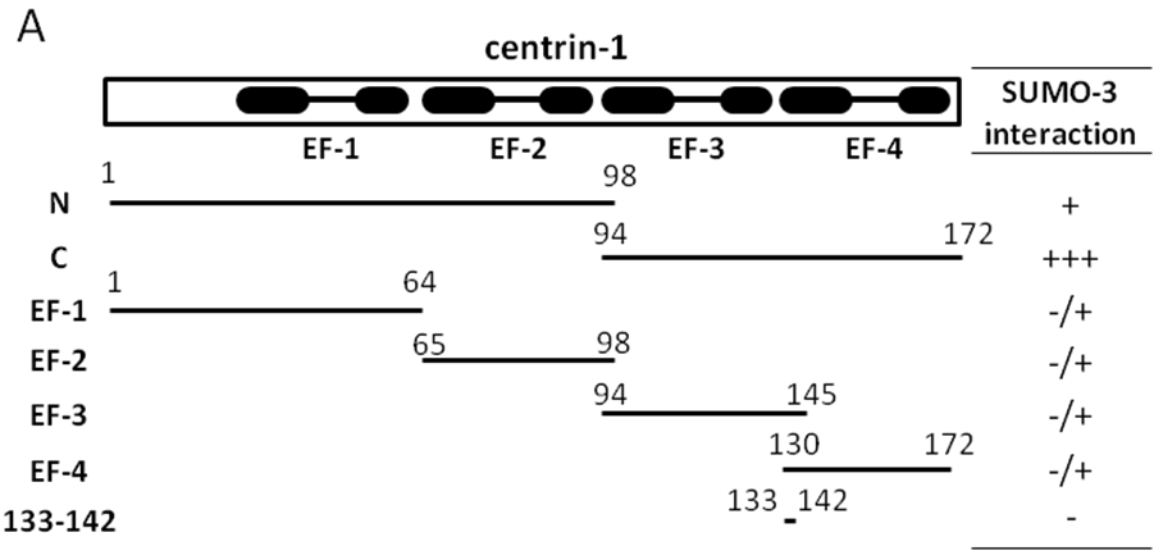


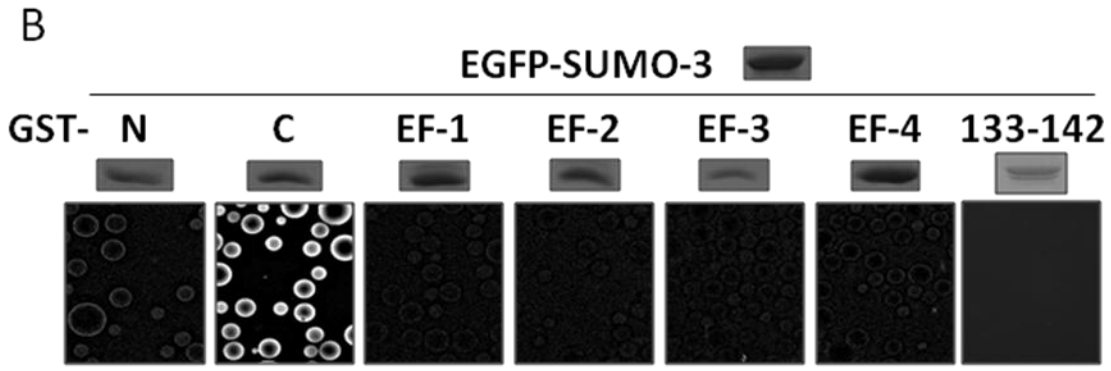
Tanaka et al. Fig. 2

E



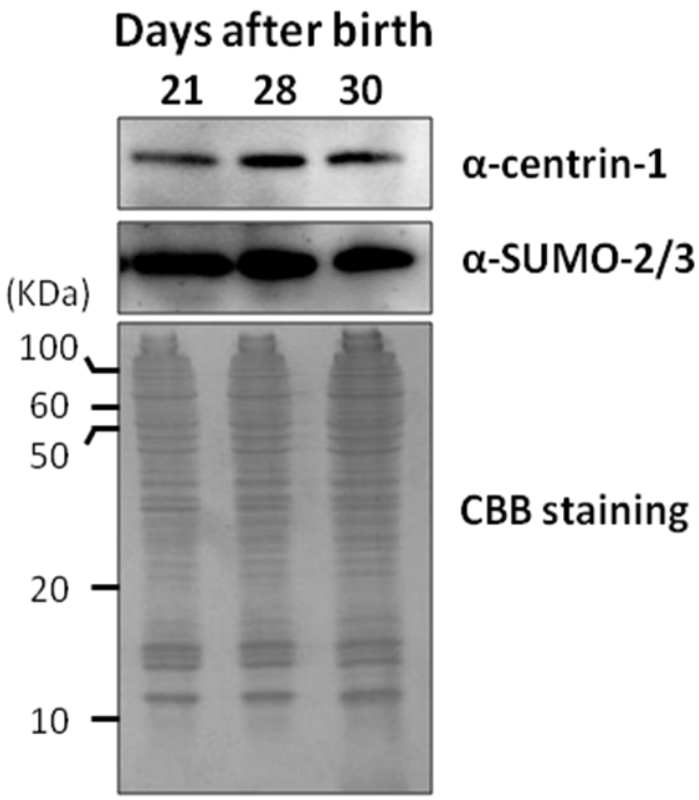
Tanaka et al. Fig. 2





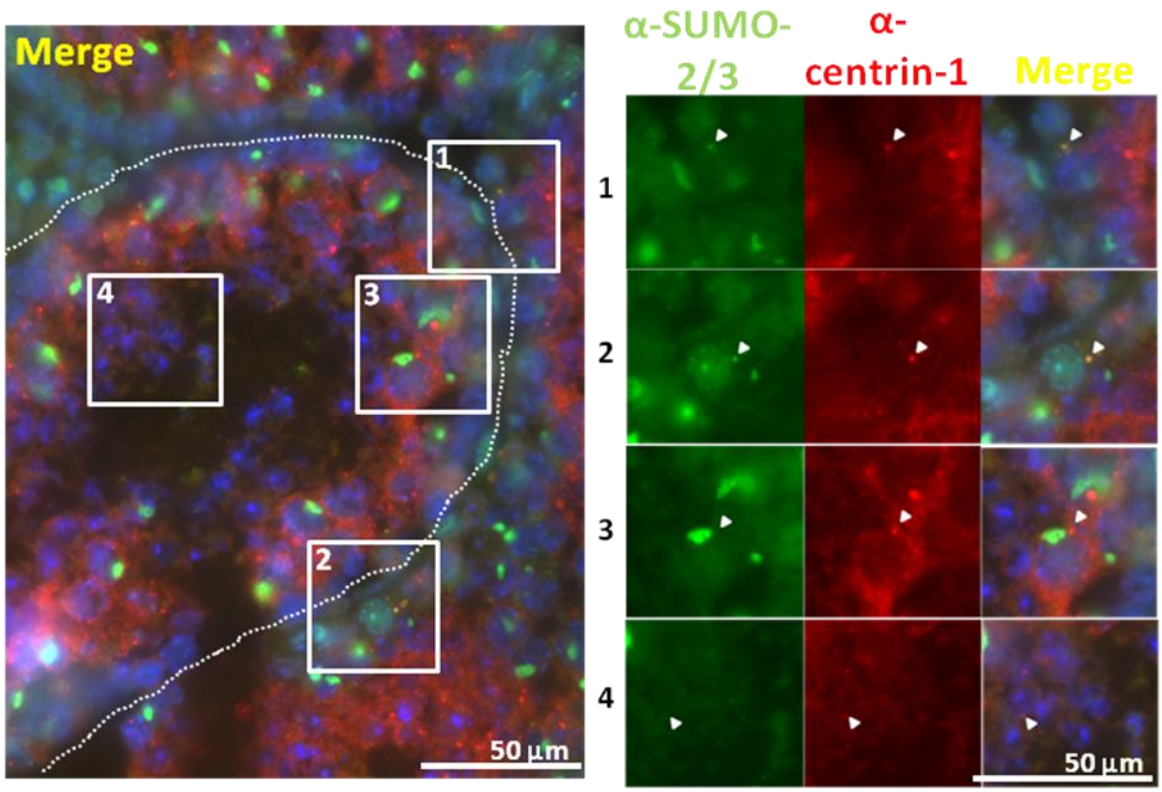
Tanaka et al. Fig. 3

A



Tanaka et al. Fig. 4

B



Tanaka et al. Fig. 4