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Sperm associated antigen 8 (SPAG8), a novel regulator of activator of CREM in testis during spermatogenesis

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ABSTRACT

cAMP response element modulator (CREM)-mediated gene expression is an essential regulatory mechanism for germ cell differentiation. CREM and its coactivator in testis, ACT, activate the transcription of many essential genes for spermatogenesis. Sperm associated antigen 8 (SPAG8) is a testis-specific component that is expressed during germ cell differentiation. In this study, we found the pattern of SPAG8 expression largely overlapped with that of ACT during spermatogenesis and verified the association of SPAG8 with ACT. Furthermore, we showed that SPAG8 enhanced the transcriptional activation of ACT-mediated CREMτ by strengthening the binding of ACT to CREMτ. These results indicate that SPAG8 acts as a regulator of ACT and plays an important role in CREM-ACT-mediated gene transcription during spermatogenesis.

Structured summary:
MINT-7892874: *CREM tau* (uniprotkb:P27699-1) *physically interacts* (MI:0915) with *ACT* (uniprotkb:Q9WTX7) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7892809: *ACT* (uniprotkb:Q9WTX7) *physically interacts* (MI:0915) with *SPAG8* (uniprotkb:B9EKF1) by *pull down* (MI:0096)
MINT-7892820, MINT-7892840, MINT-7892987, MINT-7892854: *ACT* (uniprotkb:Q9WTX7) *physically interacts* (MI:0915) with *SPAG8* (uniprotkb:B9EKF1) by *anti tag coimmunoprecipitation* (MI:0007)

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1. Introduction

As a master transcriptional activator during spermatogenesis, cAMP response element modulator (CREM) mediates the transcription of many post-meiotic genes (e.g., *Tnp1, Tnp2, Prm1* and *Prm2*) via binding the cAMP response element (CRE) in the promoters [1,2]. These gene products are responsible for chromatin remodeling and spermatid elongation. Due to alternative promoters, splicing events and translation initiation, the *Crem* gene generates a series of CREM proteins that function as either transcriptional activators (e.g. CREMτ) or transcriptional repressors [3–6]. CREMτ is the most abundant activating isoform in mouse testis at puberty and is highly expressed in spermatids [7–9]. Spermatogenesis in *Crem*-null male mice is blocked at the round spermatid stage and thereby causes sterility [7,10]. In contrast with its activation via phosphorylation and association with CBP in somatic cells, the transcriptional activity of CREM is mainly activated by its coactivator, activator of CREM in the testis (ACT) [11,12]. ACT is composed of four and a half LIM domains, each containing two zinc finger motifs. The CREM–ACT complex mediates the expression of many post-meiotic genes that are essential for normal spermatogenesis, thus providing a tissue-specific modulation mechanism for CREM transcription activity [13–16]. Although the regulatory pathway of the CREM–ACT complex is essential for spermatogenesis, the mechanism of how ACT mediates the transcription activity of CREMτ is currently unknown. Therefore, the investigation of other regulators or general transcription factors that could be associated with ACT during spermatogenesis would contribute to the understanding of this regulatory pathway.

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Sperm associated antigen 8 (SPAG8), previously designated as hSMP-1 or HSD-1, was isolated from a human testis expression library using anti-sperm antibodies obtained from the serum of an infertile woman. SPAG8 is localized in spermatids and in the head and tail of sperm in rat testis [17,18], suggesting a role for SPAG8 in germ cell differentiation. In our previous study, we found that ACT interacts with SPAG8 by yeast two-hybrid using SPAG8 as bait, which suggests that SPAG8 could be associated with ACT during spermatogenesis.

In this study, we generated a SPAG8 antibody and found that SPAG8 distribution largely overlapped with that of ACT during testis development. Furthermore, we found that SPAG8 enhanced the transcriptional activation of ACT-mediated CREMτ and the binding of ACT to CREMτ. These results indicate that SPAG8 may be in-

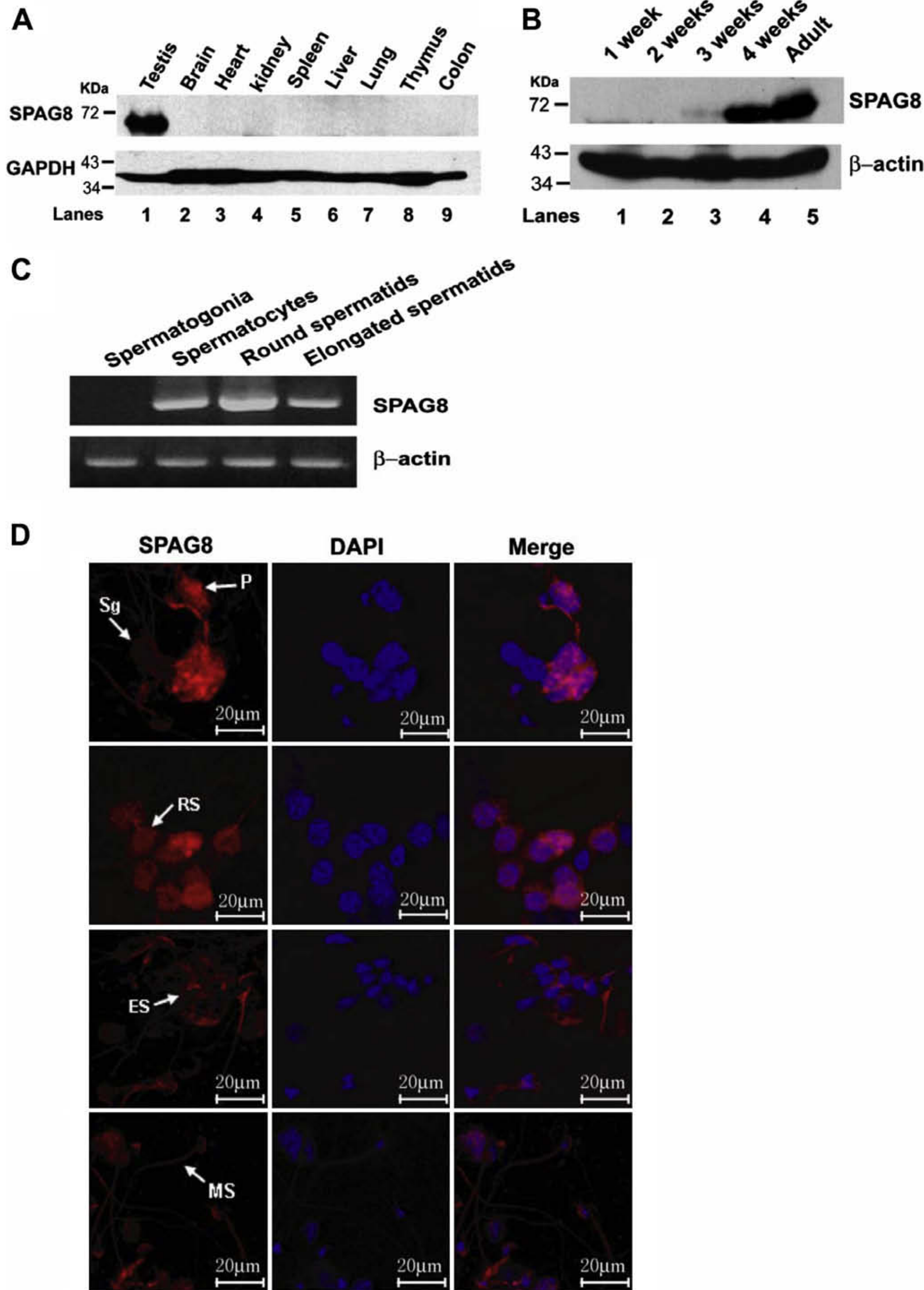

Fig. 1. The expression of SPAG8 is tissue-specific and cell stage-specific. (A) Monitoring of SPAG8 expression in various mouse tissues. Tissue extracts from Balb/c mice were analyzed with an immunoblot assay with the anti-SPAG8 antibody. GAPDH served as a loading control. (B) SPAG8 expression during testis development. Tissue extracts from the testes of mice at different ages (as indicated) were analyzed with an immunoblot assay. (C) Total RNA extracted from purified germ cells (as indicated) was analyzed by RT-PCR using *Spag8* specific primers. β-Actin served as an internal control. (D) Expression of SPAG8 in germ cells. Squash preparations were prepared from the testes of 8-week-old mice and used for immunofluorescence staining using the anti-SPAG8 antibody and DAPI. Red indicates SPAG8 immunoreactivity. Sg, spermatogonia; P, pachytene spermatocyte; RS, round spermatid; ES, elongating spermatid; MS, mature spermatozoon. Magnification, ×400.

volved in the transcriptional regulatory pathway of the CREMτ–ACT complex during spermatogenesis.

2. Materials and methods

2.1. Plasmid construction

To generate expression plasmids for HA- and Myc-SPAG8, *Spag8* coding sequence (corresponding to amino acids 1–412) was amplified using forward 5′-ATGGAGACCACCGAGTCTACA-GAGG and reverse 5′-GTCGCCCACACACACCGGTAGC primers from mouse testis cDNA and subcloned into the pcDNA6/V5-HisB and pcDNA6/myc-HisB vectors (Clontech). Various deletion fragments of SPAG8 with the Myc epitope were generated in a similar manner, resulting in Myc-SPAG8-N1(1–256), Myc-SPAG8-N2(1–140), Myc-SPAG8-N3(1–103), Myc-SPAG8-M(104–256), Myc-SPAG8-C1(256–412) and Myc-SPAG8-C2(312–412). Full-length *Act* was amplified using forward 5′-ATGACAAGTAGT-CAATTTG and reverse 5′-GTCTCTTTCTAAGCGTCAGTGTC primers from testis cDNA and subcloned into the p3xFlag-CMV-14 vector to generate an expression plasmid for Flag-ACT. Various deletion fragments of ACT with the Flag epitope were generated in a similar manner, resulting in Flag-ACTΔ1/2, Flag-ACTΔ1/2-1, Flag-ACTΔ4, Flag-ACTΔ3-4 and Flag-ACTΔ2-4 (the numbers denote the position of the LIM domains of ACT from the N-terminus to the C-terminus). For bacterial expression, full-length *Act* was subcloned into pGEX-6p-1 (Promega), generating a plasmid for the expression of a glutathione S-transferase (GST) fusion protein (GST-ACT). pCMV-6Myc-CREMτ (wt and S117A) and pGL3-5×CRE (CRE sequence derived from the somatostatin gene promoter) were kind gifts from Prof. Hiroshi Nojima (Osaka University, Japan) [13]. The CREMτ activation domain and its mutant, which contains a mutation of serine 117 to alanine, were cloned into the pGBDT7 vector with the coding sequence of the Gal4 DNA-binding domain to generate Gal4DBD-CREMτ and Gal4DBD-CREMτS117A, respectively. All construct sequences were verified by sequencing analysis.

2.2. Generation of SPAG8 antibody and Western blot

SPAG8 antibody was generated in rats immunized with a recombinant polypeptide of mouse SPAG8. The DNA fragment for residues Asn1–Asn768 of SPAG8 was subcloned into the pGEX-6P-1 plasmid. The soluble, 72-kDa GST-SPAG8(1–253) fusion protein was purified using glutathione-sepharose 4B beads. After GST was separated from GST-SPAG8 protein, the purified SPAG8 polypeptide (1 mg/ml) was used as a SPAG8 antigen, and Rat anti-SPAG8 antiserum was generated by sequential immunization with 100 μg of purified protein.

Tissues, including testes from 1-, 2-, 3- and 4-week-old and adult Balb/c mice were collected and homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. Aliquots containing 100 μg of total protein were analyzed by Western blot using anti-SPAG8 and β-actin antibodies.

2.3. Isolation of germ cells, RNA isolation and RT-PCR

Germ cells were isolated from the testes of adult Balb/c mice as described [19]. Briefly, the tunica albuginea of the mouse testis was removed. The remaining tissues were digested with 0.5 mg/ml collagenase (Gibco) in DMEM with 0.5% BSA at 32 °C for 15 min. After the addition of PBS containing 1 mM EDTA, the tubule suspension was transferred into a conical tube and left standing for 5 min to precipitate tubule fragments. The supernatant, which contained Leydig cells, was discarded. The tubules were reincubated in

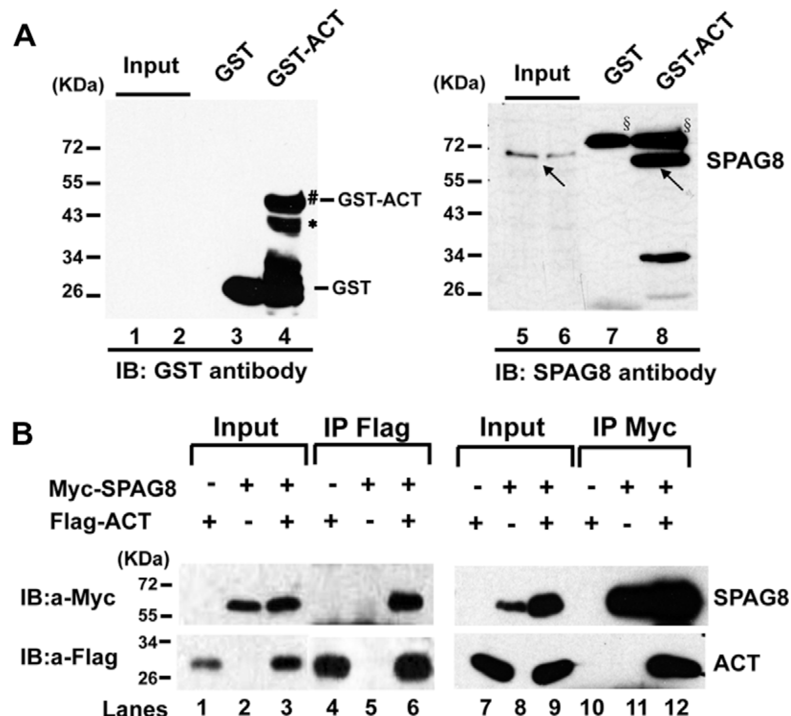

Fig. 2. Association of SPAG8 with ACT in vitro and in vivo. (A) Pull-down assay performed with testes extracts of 12-week-old mouse and GST-ACT and GST. GST protein served as the negative control. # and * indicate full-length and partially degraded GST-ACT proteins, respectively. § indicates a non-specific band, and an arrow points to the SPAG8 protein. (B) HEK293T cells were co-transfected with Flag-ACT- and Myc-SPAG8-expressing vectors. Cell lysates were coimmunoprecipitated with the appropriate anti tag antibodies as indicated. Lanes labeled "Input" were loaded with 5% of the total cell lysates.

DMEM with 0.5% BSA, dispersed by gentle pipetting in PBS containing 1 mM EDTA to remove residual Leydig cells and washed with PBS containing 1 mM EDTA. The cell suspensions were fractionated by unit gravity sedimentation on a BSA gradient. The purity of the enriched populations of spermatogonia, pachytene spermatocytes, round spermatids, and elongated spermatids was between 85% and 90%. Total RNA from isolated germ cells was extracted using the Trizol®RNA Isolation Kit (Invitrogen). cDNA products were obtained using the Reverse transcription system (Promega) and amplified by PCR with specific *Spag8* and β-actin primers.

2.4. Immunofluorescence

Seminiferous tubules were isolated from adult Balb/c mice. After incubation in 0.5 mg/ml collagenase in DMEM with 0.5% BSA for 15 min, seminiferous tubules were digested into single cells or clumps of cells. The cells were squashed onto glass slides. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Non-specific sites were blocked with 5% bovine serum albumin for 1 h. The immunofluorescence analysis was performed using the anti-SPAG8 antibody. AlexaFluor goat anti-rat IgG was used as the secondary antibody (Invitrogen).

2.5. Pull-down assay

Recombinant GST-ACT protein was purified from the *Escherichia coli* BL21 strain with glutathione-sepharose (Amersham Pharmacia). Twelve-week-old mouse testes were collected in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Roche). Following sonication and centrifugation, aliquots of total protein were mixed with purified GST-ACT and GST protein, respectively. The mixture was rotated at 4 °C overnight, and 30 μl glutathione-sepharose 4B was added. The resultant protein was subjected to Western blot analysis after extensive washing using an anti-GST antibody (Sigma) and the anti-SPAG8 antibody.

2.6. Cell culture, transfection and dual luciferase reporter assays

HEK293T cells (in DMEM, 10% FBS) were transfected with the firefly luciferase expression plasmid and the renilla luciferase expression vector pRL-TK and the indicated expression constructs (see Figs. 4 and 6) using the Engreen transfection reagent. Thirty-six hours after transfection, the cells were lysed in a passive lysis

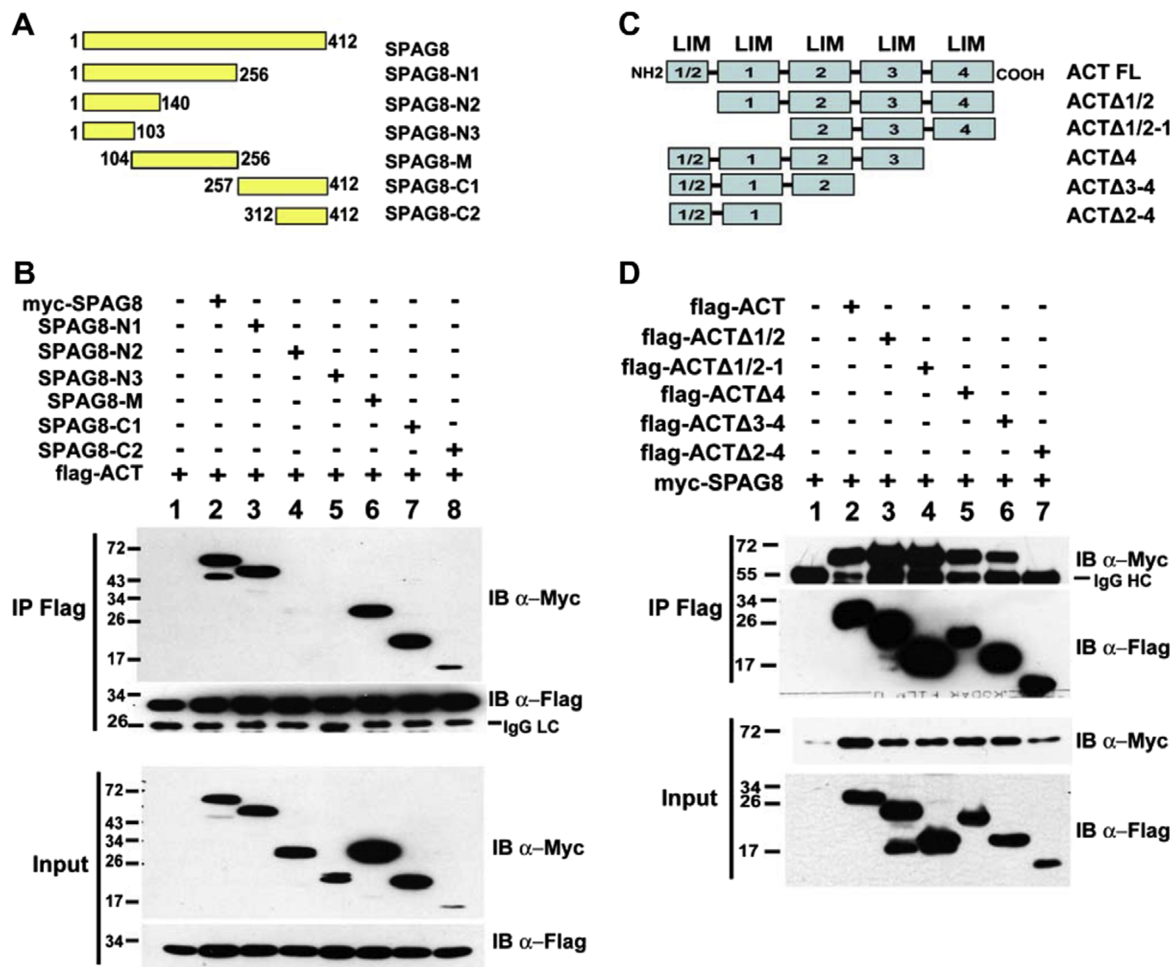

Fig. 3. Characterization of the interacting regions of SPAG8 and ACT. (A) Schematic alignment of full-length SPAG8 with the deletion fragments used in the experiments. (B) Lysates of HEK293T cells expressing Myc-tagged SPAG8 or its deletion mutants together with Flag-tagged ACT were immunoprecipitated (IP) using an anti-Flag antibody. Precipitates were immunoblotted (IB) for associated proteins using the appropriate antibodies. (C) Schematic illustration of the ACT deletion fragments used in this study. Boxes depict the LIM domains. (D) Expression plasmids of Myc-tagged SPAG8 and Flag-tagged ACT or its deletion mutants were co-transfected in HEK293T cells and immunoprecipitated (IP) with an anti-Flag antibody. Immunoprecipitated fractions were analyzed by immunoblotting (IB) using an anti-Myc antibody or an anti-Flag antibody.

buffer and assayed for firefly and renilla luciferase activities using the Dual Luciferase Assay System (Promega). The results were normalized against renilla luciferase activity. The data shown are from at least three independent transfection experiments with a variation of less than 12% between the measurements.

2.7. Co-immunoprecipitation assays

HEK293T cells were transfected with the appropriate expression vectors and collected in 1 ml EBC (50 mM Tris–HCl [pH 7.5], 170 mM NaCl, 0.5% NP-40, 50 mM NaF) containing 1 mM PMSF and a protease inhibitor cocktail (Roche). The lysates were centrifuged at 17 400×g for 15 min at 4 °C. The supernatants were then precleared for 30 min with 50 µl Protein A-Sepharose (Roche). The supernatants were subsequently incubated with the appropriate antibodies with rotation overnight at 4 °C. Protein A-Sepharose was then added to precipitate this mixture. After extensive washing, the precipitated proteins were subjected to Western blot analysis using the appropriate antibodies.

3. Results

3.1. Expression of SPAG8 is testis-specific and cell stage-specific during spermatogenesis

To assess the tissue-specific function of the SPAG8 protein, it is essential to determine its tissue distribution. An anti-SPAG8 polyclonal antibody was generated to analyze the tissue distribution of the SPAG8 protein. The SPAG8 protein was mainly expressed in the testis compared with other tissues examined (Fig. 1A, lane 1). To determine the pattern of SPAG8 expression in the developing mouse testis, extracts from 1- to 4-week-old and adult Balb/c mouse testes were analyzed by Western blot. The results showed that the expression of the SPAG8 protein was very low at 3 weeks (Fig. 1B, lane 3); subsequently, levels were markedly elevated by the fourth week and into adulthood (Fig. 1B, lanes 4 and 5).

We further examined *Spag8* expression in purified germ cells by RT-PCR. The *Spag8* transcript was produced in spermatocytes and spermatids, but was absent in spermatogonia (Fig. 1C). Immunoflu-

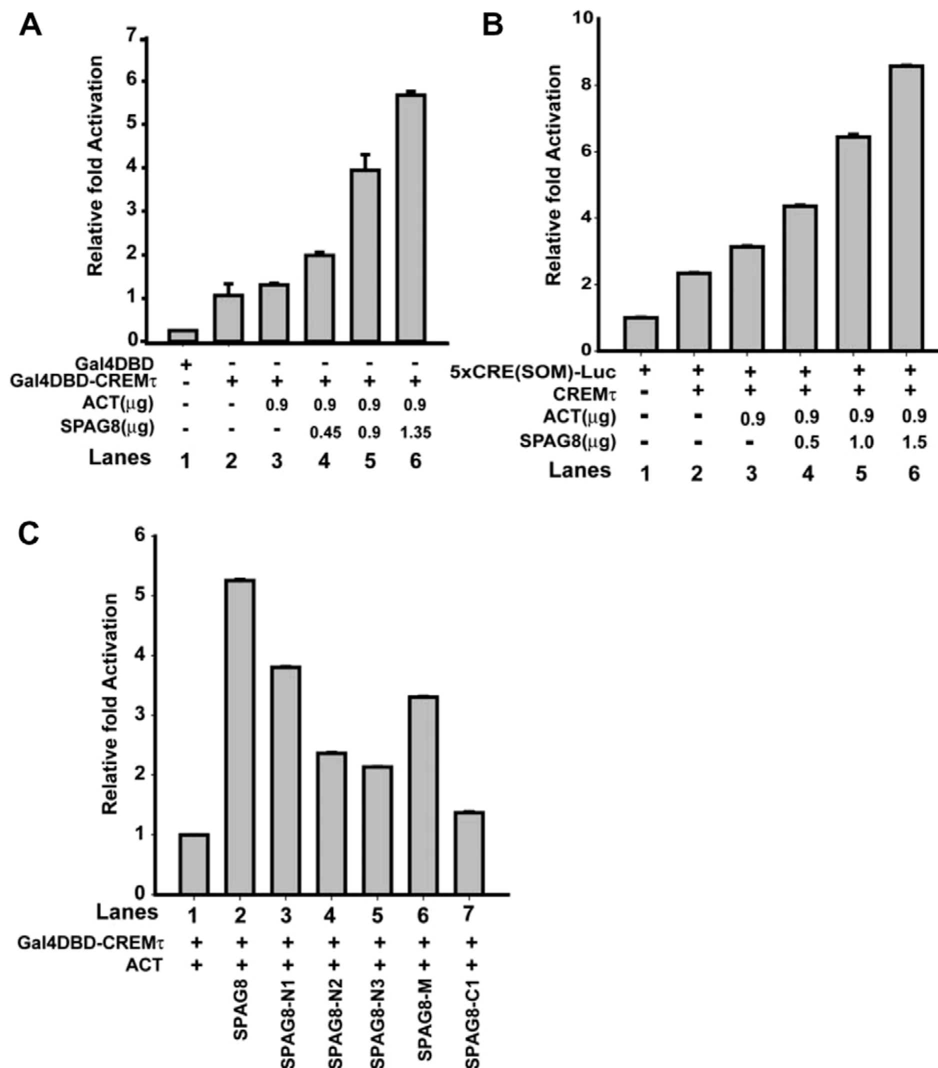

Fig. 4. SPAG8 enhances ACT-mediated CREMτ transcriptional activation. (A) Expression vectors for Gal4DBD-CREMτ and ACT and increasing amounts of the SPAG8-expressing vector were co-transfected with the reporters in HEK293T cells. Cell lysates were subjected to dual luciferase assays. (B) Luciferase assays of HEK293T cells that were co-transfected with expression vectors for ACT and CREMτ and a CRE-Luc reporter, which contains somatostatin CRE, with increasing amounts of the SPAG8-expressing vector. (C) HEK293T cells were co-transfected with expression vectors for the SPAG8 deletion mutant-expressing vectors and different expression constructs as shown. Cell lysates were subjected to luciferase assays. All data are shown as the mean ± standard deviation from three independent experiments. The results were normalized to the *Renilla* luciferase activities obtained from pRL-TK, which served as a transfection control.

orescence assays of isolated germ cells showed that SPAG8 localized to the nucleus and cytoplasm of spermatocytes and round spermatids (Fig. 1D, upper two panels). In elongated spermatids, immunostaining signals of SPAG8 were cytoplasmic, whereas no signal was observed in the nucleus (Fig. 1D, second panel from the bottom). In addition, SPAG8 expression appeared to be concentrated in the region of the acrosome and the middle piece of the tail in mature spermatozoa (Fig. 1D, bottom panel). In contrast, SPAG8 immunoreactivity was absent in spermatogonia (Fig. 1D, upper panel). Taken together, this spatial and temporal expression pattern of SPAG8 suggests that SPAG8 functions in a germ cell type- and stage-specific manner during spermatogenesis.

3.2. Interaction of SPAG8 and ACT in vitro and in vivo

ACT is initially expressed in pachytene spermatocytes. During spermatid elongation, it localizes to the nucleus of round spermatids but exits the nucleus to the cytoplasm as cells differentiate to elongated spermatids [20,21]. This pattern of ACT expression coincides with that of SPAG8, indicating that the two proteins display a coupled intercellular localization in male germ cells. Based on the result of a yeast two-hybrid assay in which SPAG8 was used as bait and on the distribution patterns of ACT and SPAG8 during testis development, we performed an in vitro pull-down assay using purified GST-ACT to precipitate testis proteins. Although the GST-ACT fusion protein was slightly degraded (indicated by *), full-length GST-ACT was clearly detected (indicated by #) and deemed sufficient to perform the pull-down assays (Fig. 2A, lanes 3 and 4). The SPAG8 protein was associated with GST-ACT in vitro (lane 8), but not with GST alone (lane 7). We further determined the association between SPAG8 and ACT in HEK293T cells by co-immunoprecipitation assays. The results showed that SPAG8 was associated with ACT in the immunoprecipitated complexes from co-transfected cells (Fig. 2B, lanes 6 and 12). We could not perform endogenous immunoprecipitation experiments because the anti-ACT antibody showed non-specific detection of proteins in a testis extraction (data not shown).

3.3. Amino acids 140–412 of the SPAG8 sequence and the three C-terminal LIM domains of ACT are involved in the interaction between SPAG8 and ACT

To characterize the regions of SPAG8 and ACT responsible for the interaction, we performed deletion assays and cell immunoprecipitation assays. We constructed deletion mutants of SPAG8, as shown in the schematic drawing (Fig. 3A). The interaction between ACT and the SPAG8 deletion mutants was investigated by coimmunoprecipitation assays. Immunoprecipitation of the Flag-tagged ACT coprecipitated SPAG8-N1(1–256), SPAG8-M(103–256), SPAG8-C1(257–412) and SPAG8-C2(312–412) (Fig. 3B, lanes 6–8). In contrast, no coprecipitation of SPAG8-N2(1–140) or SPAG8-N3(1–103) was observed (Fig. 3B, lanes 4 and 5). This deletion analysis demonstrated that two domains in SPAG8, the first within 140–256 and the second within 257–412, are involved in its interaction with ACT. Either domain could interact with ACT without the other. To assess which LIM domains of ACT are responsible for its interaction with SPAG8, we further generated some deletion mutants of ACT (Fig. 3C) and performed a co-immunoprecipitation assay. The ACT fragments that lacked the N-terminal half LIM domain and the first full LIM domain (Flag-ACTΔ1/2-1) or the C-terminal third and fourth LIM domains (Flag-ACTΔ3/4) bound to SPAG8 (Fig. 3D, lanes 4 and 6), while the ACT fragments that lacked the three C-terminal LIM domains (Flag-ACTΔ2-4) were completely unable to interact with SPAG8 (lane 7). Therefore, it is possible that the second LIM domain is essential for the binding of ACT to SPAG8.

3.4. SPAG8 enhances the transcriptional activation of ACT-mediated CREMτ

Because ACT is believed to function as a trans-activator of CREM during spermatogenesis [12,13], we wanted to determine whether SPAG8 can affect ACT-mediated CREMτ transcriptional activation. Thus, we performed dual luciferase reporter gene assays by cotransfecting HEK293T cells with a reporter plasmid containing a Gal4DBD-CREMτAD binding site upstream of the firefly luciferase cDNA with or without the Myc-SPAG8-expressing vector. We found

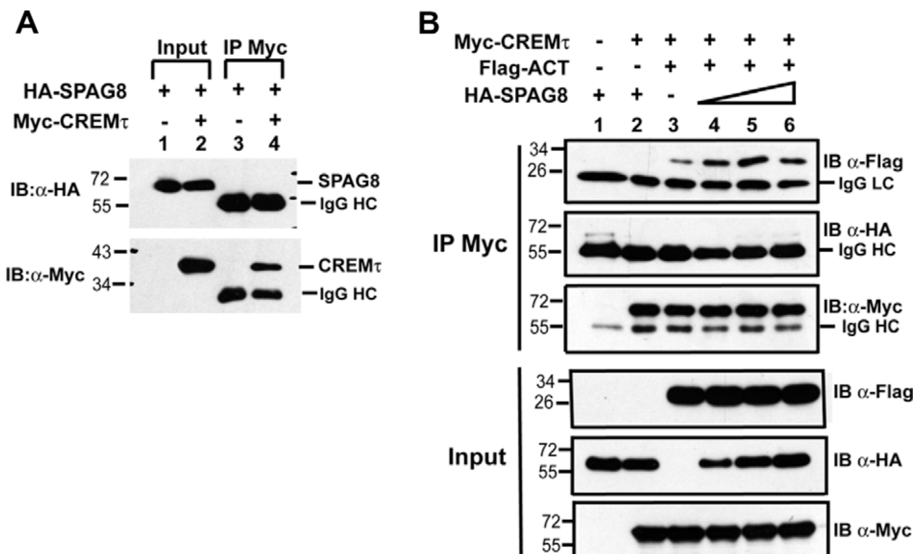

Fig. 5. SPAG8 enhances the binding of ACT to CREMτ in HEK293T cells. (A) HEK293T cells were co-transfected with Myc-CREMτ and HA-SPAG8 expression vectors and immunoprecipitated (IP) with an anti-Myc antibody. Immunoprecipitated fractions were analyzed by immunoblotting (IB) using an anti-HA antibody and an anti-HA antibody, respectively. (B) Myc-CREMτ and Flag-ACT expression vectors and increasing amounts of the SPAG8-expressing vector were transfected in HEK293T cells and immunoprecipitated (IP) with an anti-Myc antibody. The 55-kDa band in the second and third panel from the top corresponds to the heavy chain of immunoglobulin.

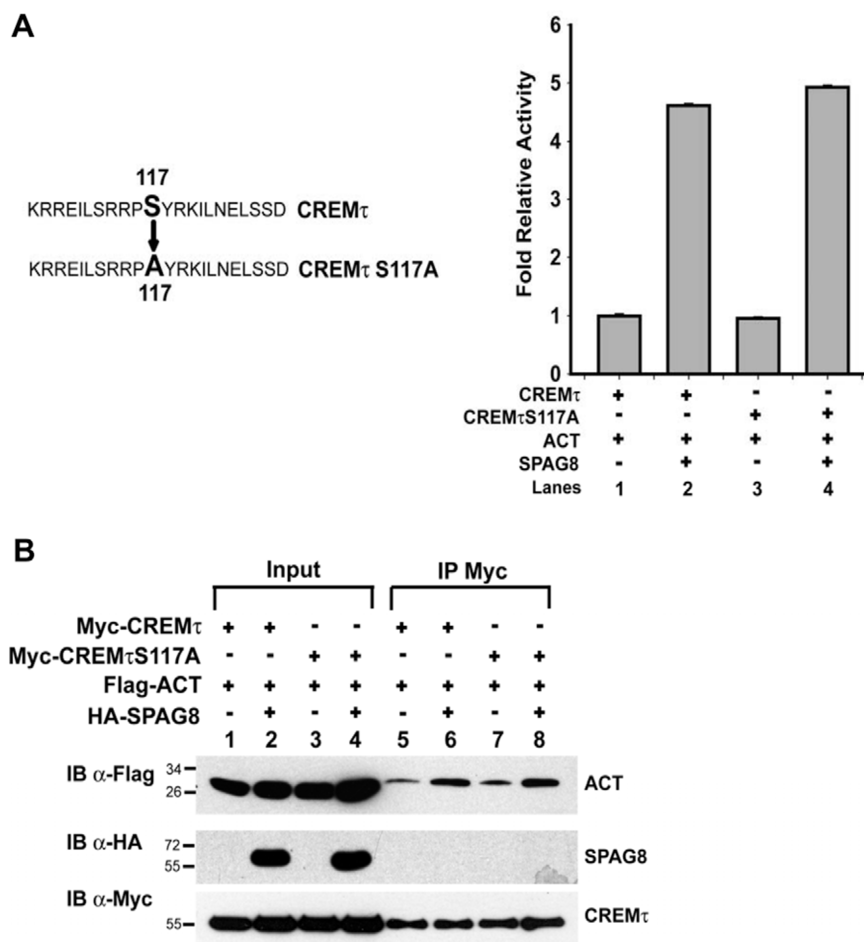

Fig. 6. Phosphorylation of CREMτ Ser117 is not a requirement for SPAG8 activity in cultured cells. (A) SPAG8 enhances ACT-mediated CREMτ transcriptional activation independent of Ser117 in HEK293T cells. *Left*, Sequence of the peptide within the CREMτ activation domain, which contains Ser117, compared with its mutated counterpart (CREMτAla117). *Right*, Luciferase assay of HEK293T cells transfected with expression vectors for Gal4DBD-CREMτ or Gal4DBD-CREMτS117A with or without different expression plasmids as shown. (B) HEK293T cells were transfected with expression vectors for Myc-CREMτ or Myc-CREMτS117A and Flag-ACT together with HA-SPAG8. Immunoprecipitation (IP) was performed with an anti-Myc antibody and precipitates were immunoblotted (IB) for associated proteins using anti-HA, anti-Flag and anti-Myc antibodies.

that ACT-activated transcription was markedly enhanced by SPAG8 in a dose-dependent manner (Fig. 4A). Similar results were obtained with full-length CREMτ on a CRE-driven somatostatin reporter promoter (Fig. 4B). These results indicated that SPAG8 possibly enhanced ACT-regulated CREM transcriptional activation. To further determine which region of SPAG8 was responsible for the transcription activation property, luciferase assays with deletion mutants of the SPAG8 expression plasmids were performed. The results of this analysis showed that various regions of SPAG8 contributed differentially to the transcriptional activation function of SPAG8. Amino acids 103–256 of the SPAG8 sequence appeared to play the primary role in activation (Fig. 4C, lane 6). In contrast, the C-terminal region (residues 257–412 of SPAG8) seemed to play a minor role (lane 7). However, none of these deletions demonstrated the complete activity of the intact protein, indicating that the structural integrity of SPAG8 is necessary to enhance the transcriptional activation of ACT.

3.5. SPAG8 markedly enhances the binding of ACT to CREMτ

ACT interacts with CREMτ in spermatids [12]. Thus, we investigated whether SPAG8 associates with CREMτ. To test this theory, we co-transfected HEK293T cells with HA-SPAG8 and Myc-CREMτ expression vectors and performed a co-immunoprecipitation assay. The result of this assay revealed that SPAG8 did not directly interact with CREMτ (Fig. 5A). However, when HEK293T cells were co-transfected with Myc-CREMτ- and Flag-ACT-expressing vectors together with increasing amounts of the HA-SPAG8-expressing vector, the amount of Flag-ACT coprecipitated by Myc-CREMτ increased with increasing HA-SPAG8 expression (Fig. 5B, lanes 3–6, upper panel). In contrast, no precipitation of HA-SPAG8 was observed in the presence or absence of Flag-ACT (Fig. 5B, lanes 2 and 4–6, second panel from the upper). These results indicate that although SPAG8 cannot form a complex with ACT and CREMτ, SPAG8 has the potential ability to enhance the binding of ACT to CREMτ.

3.6. The transcriptional activity of ACT and the binding of ACT to CREMτ, which is enhanced by SPAG8, are independent of CREMτ Ser117 phosphorylation

Phosphorylation of Ser117 within the CREMτ is essential for its association with CBP in somatic cells, whereas ACT activates the transcriptional activity of CREMτ in a phosphorylation- and CBP-independent manner in germ cells [12,22]. We investigated whether this function of SPAG8 is dependent on the phosphorylation of CREMτ Ser117. To test this, we converted Ser117 to alanine to construct the Gal4DBD-CREMτS117A and Myc-CREMτS117A expression vector (Fig. 6A, left panel) and performed a luciferase assay and co-immunoprecipitation experiment. The results of the luciferase assay showed that SPAG8 enhanced the transcriptional

activation of ACT-medicated CREMτ Ser117A (Fig. 6A, right panel, lane 4). The results of co-immunoprecipitation experiment showed that the binding of ACT to Myc-CREMτS117A was also markedly enhanced by SPAG8 (Fig. 6B, lane 8, upper panel). These results indicate that the transcriptional activation of SPAG8 on ACT-mediated CREMτ is not dependent on the phosphorylation of CREMτ Ser117 in cultured cells.

4. Discussion

CREM-mediated gene expression is an essential regulatory pathway for the spermiogenic program. CREM and its coactivator, ACT, work together to activate the expression of many essential post-meiotic genes [2,3,9]. However, very few studies have been performed to elucidate the other factors involved in the complicated transcriptional regulation that occurs during spermatogenesis. In the present study, we found that the SPAG8 protein is specifically and highly expressed in the testis, which coincides with its mRNA expression, as demonstrated by our previous work [18]. SPAG8 expression in mouse testis occurs initially from the third post-natal week, at which time late pachytene spermatocytes and round spermatids accumulate. This result is in accordance with those of our cell immunofluorescence assay, in which SPAG8 was expressed from the spermatocyte to the mature sperm stage. The distribution of SPAG8 in spermatids largely overlaps with that of ACT [15,20,23], indicating that SPAG8 may associate with ACT during spermatogenesis. However, SPAG8 expression in germ cells is not restricted to cell stages that express ACT. We found that SPAG8 is also expressed in the head and middle piece of the tail of the mature spermatozoon where ACT is absent, indicating that SPAG8 has other roles in mature spermatozoa, as described previously [24,25]. For example, Cheng et al. showed that a SPAG8 antibody inhibits the mouse acrosome reaction and sperm-zona pellucida binding [25].

ACT can powerfully activate the transcriptional activity of CREMτ by interacting with it [2,5]. We also found that SPAG8 enhances the transcriptional activation of ACT-regulated CREMτ in cultured cells. This enhanced transcriptional activation may be mainly due to the ability of SPAG8 to remarkably enhance the binding of ACT to CREMτ. SPAG8 interacts with ACT, but not with CREMτ, whether CREMτ is in complex with ACT or not, indicating that SPAG8 does not directly bind to the CREMτ–ACT complex. However, the underlying mechanism of how SPAG8 regulates the binding of ACT to CREMτ remains unknown. We speculate that it is possibly related to nuclear transport in a microtubule-dependent manner. First, the pattern of SPAG8 expression in germ cells indicates a possible shuttling activity for this protein, which can be supported by our previous study that SPAG8 has a close relationship with microtubules and its location in CHO cells that stably express SPAG8 changes with the progress of the cell cycle [26]. Furthermore, SPAG8 has been confirmed to associate with RanBPM [27]. RanBPM is a Ran-binding protein in the microtubule-organization center, which has a role in Ran-dependent nuclear transport [28]. SPAG8 may have a similar role to transport ACT into the nucleus of round spermatids, although further experiments are required to validate this hypothesis.

In conclusion, the present study demonstrated that SPAG8 is a novel regulator that enhances the transcriptional activation of ACT-mediated CREMτ. This finding allows us to more deeply understand the complicated transcriptional regulation that occurs during spermatogenesis.

Acknowledgments

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