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Germ cell-specific gene 1 targets testis-specific poly(A) polymerase to the endoplasmic reticulum through protein–protein interactions

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Abstract **Testis-specific poly(A) polymerase (TPAP) is a cytoplasmic poly(A) polymerase that is highly expressed in round spermatids. We identified germ cell-specific gene 1 (GSG1) as a TPAP interaction partner protein using yeast two-hybrid and coimmunoprecipitation assays. Subcellular fractionation analysis showed that GSG1 is exclusively localized in the endoplasmic reticulum (ER) of mouse testis where TPAP is also present. In NIH3T3 cells cotransfected with TPAP and GSG1, both proteins colocalize in the ER. Moreover, expression of GSG1 stimulates TPAP targeting to the ER, suggesting that interactions between the two proteins lead to the redistribution of TPAP from the cytosol to the ER.**

Structured summary:
MINT-6168263:
Gsg1 (Q8R1W2), *TPAP* (Q9WVP6) and *Calmegin* (P52194) *colocalize* (0403) by *cosedimentation* (0027)
MINT-6168204, MINT-6168178:
Gsg1 (Q8R1W2) and *TPAP* (Q9WVP6) *colocalize* (0403) by *fluorescence microscopy* (0416)
MINT-6167930:
Gsg1 (Q8R1W2) *physically interacts* (0218) with *TPAP* (Q9WVP6) by *two-hybrid* (0018)
MINT-6168112, MINT-6168011, MINT-6168054:
Gsg1 (Q8R1W2) *physically interacts* (0218) with *TPAP* (Q9WVP6) by *coimmunoprecipitation* (0019)
MINT-61668069, MINT-6168101:
Gsg1 (Q8R1W2)*physically interacts* (0218) with *TPAP* (Q9WVP6) by *pull-down* (0096)
MINT-6168218:
Gsg1 (Q8R1W2) and *GRP78* (P20029) *colocalize* (0403) by *fluorescence microscopy* (0416)
MINT-6168381:
TPAP (Q9WVP6) and *GRP78* (P20029) *colocalize* (0403) by *fluorescence microscopy* (0416)

Keywords: Cytoplasmic polyadenylation; Spermatogenesis; Membrane protein

1. Introduction

Polyadenylation of eukaryotic mRNA is a significant event for post-transcriptional and translational regulation involved in export from the nucleus to the cytoplasm, as well as stability and translation of mRNA [1–4]. Since transcriptional block is essential for the formation of germ cells, translational control by polyadenylation in the cytoplasm is particularly important in germ cell differentiation. In *Xenopus* oocyte maturation, cytoplasmic polyadenylation mediated by cytoplasmic polyadenylation element binding protein (CPEB) induces the translation of maternal mRNA [5]. In addition to CPEB, the CPEB- and CPSF-binding protein, symplekin, and the cytoplasmic PAP, xGLD-2, act in the cytoplasmic polyadenylation machinery [6]. In mouse testis, another novel member of the CPEB protein family (CPEB2) and a homolog of xGLD-2 (mGLD-2) have been identified [7,8], suggestive of similar CPEB-dependent cytoplasmic polyadenylation during spermatogenesis. However, in contrast to *Xenopus* oocytes, mouse testis contains another cytoplasmic polymerase, designated 'testis-specific poly(A) polymerase' (TPAP) or PAP β, which is highly expressed in round spermatids and involved in the addition of poly(A) at the 3′-ends of some mRNAs in haploid germ cells [9]. TPAP, but not mGLD-2, contains RNA-binding and S/T-rich domains. GLD-2 localizes in both the cytoplasm and nucleus of somatic, testicular, and cultured cells [8]. In contrast, TPAP is expressed only in testis, and localizes predominantly in the cytoplasm [9,10]. Therefore, TPAP-mediated cytoplasmic polyadenylation possibly occurs via a testis-specific and CPEB-independent mechanism.

In TPAP-deficient mice, expression of haploid-specific genes required for morphogenesis of germ cells is impaired, and poly(A) tails of specific transcription factor mRNAs of round spermatids are not elongated completely. Consequently, these mice are infertile due to spermatogenesis arrest [11]. Mice overexpressing TPAP display normal spermatogenesis and fertility, and the mRNA sizes of the transcription factors are unaltered [12], suggestive of limiting regulatory factors that may act via interactions with TPAP.

In this study, we identify germ cell-specific gene 1 protein (GSG1) as a TPAP interaction partner protein. The 40 kDa GSG1 protein consists of 365 amino acids. GSG1 mRNA is expressed on day 24 of life in mouse testis [13], coincident with the expression of TPAP mRNA. We show that GSG1 interacts with TPAP, leading to redistribution of TPAP from the cytosol to the endoplasmic reticulum.

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Abbreviations: TPAP, testis-specific poly(A) polymerase; GSG1, germ cell-specific gene 1 protein; ER, endoplasmic reticulum

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2. Materials and methods

2.1. Yeast two-hybrid screen

The Matchmaker GAL-4-based two-hybrid system (Clontech) was employed, using the full sequence of mouse TPAP as bait. An adult mouse testis library prepared by fusion to the GAL4 activation domain was purchased from Clontech. Yeast two-hybrid screening was performed by mating with the yeast strains, Y187 and AH109, according to the manufacturer's instructions.

2.2. Soluble and insoluble fractionation of nucleus and cytoplasm from mouse testis

Mouse testis (0.2 g) was homogenized in 2 ml of extraction buffer (10 mM HEPES/KOH, pH 7.4, 15 mM KCl, 1 mM EDTA, 0.25 M sucrose, 0.5 mM DTT, 0.5 mM PMSF) with a Dounce homogenizer. The homogenate was centrifuged at $2500 \times g$ for 10 min, and the resulting supernatant re-centrifuged at $100000 \times g$ for 30 min. The supernatant from this step contained the soluble cytoplasmic fraction. The remaining pellet, suspended in 1.6 ml of extraction solution with 1% Triton X-100, contained the insoluble cytoplasmic fraction. The pellet obtained from the initial centrifugation was suspended in 2 ml of extraction buffer, overlaid onto 2.8 ml of extraction buffer containing 0.5 M sucrose, and centrifuged at $2500 \times g$ for 10 min. The resulting pellet was suspended in 0.4 ml of extraction buffer containing 0.5 M NaCl, and centrifuged at $20000 \times g$ for 10 min. The supernatant solution contained the soluble nuclear fraction. The final pellet, which was washed with 1 ml of extraction buffer and suspended in 0.4 ml of extraction buffer with 1% Triton X-100, contained the insoluble nuclear fraction.

2.3. Preparation of recombinant cDNA constructs

For expression of GST-TPAP or its derivatives in NIH3T3 cells, the corresponding cDNA sequences were cloned into pEBG [14]. FLAG-GSG1 and FLAG-TPAP were obtained by cloning the coding sequences of GSG1 and TPAP into p3XFLAG-CMV-7.1 (Sigma). A plasmid expressing GSG1-EGFP was generated by cloning the GSG1 coding region into pEGFP-N1 (Clontech).

2.4. Antibodies

The anti-GSG1 antibody synthesized using an oligopeptide containing residues 233–244 of GSG1 coupled to bovine serum albumin was provided by Peptron. The anti-TPAP antibody was generated according to a previous report [9]. The anti-calmegin monoclonal antibody was kindly provided by Dr. Y. Nishimune. Antibodies specific for GST, GRP78, GFP, hnRNP C1/C2, pERK, and eIF4E were purchased from Santa Cruz, while the FLAG antibody was obtained from Sigma. The FITC- or Cy3-conjugated secondary antibody was from Jackson Immunoresearch. The anti-GM130 antibody was acquired from Abcam.

2.5. Coimmunoprecipitation

NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells ($\sim 1 \times 10^8$) were transfected with DNA constructs (1 μg) using Lipofectamine reagent (Invitrogen). Coimmunoprecipitations were performed as described previously [14]. Similar, coimmunoprecipitation assays using lysates from mouse testes (0.2 g) were also performed with anti-TPAP and anti-GSG1 antibodies.

2.6. Confocal laser scanning microscopy

NIH/3T3 cells were transfected with cDNA (3 μg) expressing GSG1-EGFP and/or 3XFLAG-TPAP. Transfected cells were visualized on a Zeiss LSM510 meta confocal microscope after the treatment of anti-GRP78, GFP, GM130 and FLAG antibodies, followed by the FITC- or Cy3-conjugated secondary antibody, as described previously [15].

2.7. Immunocytochemistry

Glass slides fixed with mouse spermatogenic cells were prepared as described previously [9].The slides were treated with 0.5% Triton-100 in phosphate-buffered saline (PBS) for 5 min, washed three times with PBS, incubated for 60 min with blocking solution containing 2% donkey serum and 0.03% TritonX-100 in TBS (150 mM NaCl, 10 mM Tris–Cl, pH 7.5). Anti-GSG1 antibody and anti-TPAP antibody were diluted in the blocking solution and applied to the slides for 3 h. The slides were washed three times with 0.03% TritonX-100 in TBS, treated with Cy3- and FITC-conjugated secondary antibody for 2 h, and washed with 0.03% TritonX-100 in TBS. Confocal images were acquired with Zeiss LSM510 meta.

2.8. Membrane flotation assay

The membrane flotation assay was performed with mouse testes (0.2 g), as described previously [16]. Fractions were collected, and 8% of each fraction was immunoblotted with antibodies.

3. Results

3.1. Identification of GSG1 as an interacting partner protein of TPAP

As an initial step in analyzing TPAP-mediated cytoplasmic polyadenylation, we searched for interacting proteins. A GAL4-based yeast two-hybrid screen was performed using the full amino acid sequence of mouse TPAP as bait. One of the interacting clones encodes mouse GSG1. GSG1 mRNA is expressed 24 days after birth in mouse testis, but not in 16-day-old testis [13]. However, its function is not known at present. Sequence analysis of GSG1 using HMMTOP 2.0 revealed the presence of four putative membrane helix motifs (Fig. 1A). The TPAP-binding sequence, identified from the yeast two-hybrid screen, resides in the C-terminal region of GSG1 that is devoid of helix motifs. We examined whether GSG1 exists in the membrane fraction of mouse testis. We found that the majority of GSG1 exists in the insoluble fraction of the cytoplasm of adult mouse testis (Fig. 1B), suggestive of a membrane protein.

Expression of GSG1 at the protein level has not been known, while expression of TPAP protein in round spermatids and pachytene spermatocytes during testis development has been reported [9]. To examine how GSG1 protein is expressed during testis development, we performed immunoblot analyses. GSG1 protein as well as TPAP protein was detected in 21-, 26-, 32-old mouse testis, but not in 15-day-old mouse testis (Fig. 1C). This result suggests that both GSG1 and TPAP proteins could be expressed coordinately during mouse testis development and spermatogenesis.

3.2. Interactions of TPAP and GSG1 in vivo

To determine whether interactions between mouse TPAP and GSG1 occur in vivo, we performed a coimmunoprecipitation experiment using NIH/3T3 cells cotransfected with GST-TPAP and FLAG-GSG1 DNA. Total lysates from transfected cells were immunoprecipitated with an anti-FLAG antibody, and coimmunoprecipitated materials were immunoblotted with an anti-GST antibody. TPAP was present in GSG1 immunoprecipitates (Fig. 2B). The in vivo data suggest that TPAP–GSG1 interactions occur in mammalian cells.

Next, we performed coimmunoprecipitation assays to determine whether endogenous TPAP and GSG1 interact in mouse testis. Lysates of testes from 6-week-old mice were immunoprecipitated with anti-TPAP antibody, and blotted with an anti-GSG1 antibody. The data show that the two proteins interact within the testis (Fig. 2C).

To delineate the binding domain in TPAP, we generated several truncated mutants of the protein. Mutant proteins were tested for their ability to bind GSG1 by coimmunoprecipitation analyses (Fig. 2A and D). When coimmunoprecipitation signals were compared to the corresponding proteins present

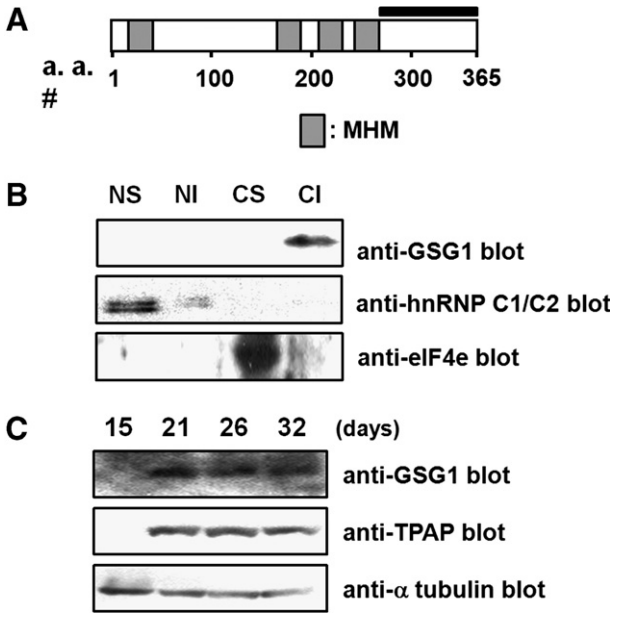

Fig. 1. Characterization of GSG1 protein. (A) Schematic diagram presenting the structure of GSG1. The sequence (265–365) initially identified from the yeast two-hybrid screening is presented as solid bars. MHM, putative membrane helix motif. (B) Cellular location of GSG1. Nuclear soluble fraction (NS), nuclear insoluble fraction (NI), cytoplasmic soluble fraction (CS), and cytoplasmic insoluble fraction (CI) from mouse testes tissues were separated. Proteins from the same proportion of each fraction were subjected to immunoblotting using an anti-GSG1 antibody. Each fraction was probed with hnRNP C1/C2 antibody as a nuclear marker and eIF4E antibody as a cytosolic marker. (C) Developmental expression of GSG1 protein in mouse testis. Testis lysates from 15-, 21-, 26-, and 32-day-old mice were used for immunoblot analysis. TPAP was also probed with anti-TPAP antibody. α-tubulin is a loading control.

in total lysates, the N-terminal 365 residues (catalytic domain of TPAP) and the 366–508 fragment (RNA-binding region of TPAP) displayed strong and weak binding to GSG1, respectively. In contrast, we observed little interaction between the C-terminal serine/threonine rich region of TPAP and GSG1. Although FLAG-GSG1 was expressed as two forms, both forms reacted with an anti-GSG1 antibody (data not shown). Since phosphorylation is important in regulating protein–protein interactions [15], we examined whether binding between TPAP and GSG1 was phosphorylation-dependent. Prior to immunoprecipitation, cell lysates cotransfected with GST-TPAP and FLAG-GSG1 were treated with non-specific protein phosphatase (Fig. 2E). However, this treatment did not affect interactions between TPAP and GSG1. This phosphorylation-independent interaction is consistent with the finding that GSG1 does not bind to the serine/threonine-rich region of TPAP containing several putative phosphorylation sites (Fig. 2D).

3.3. Confocal microscopy shows the colocalizaion of TPAP and GSG1 in spermatogenic cells

To examine whether TPAP and GSG1 are colocalized in spematogenic cells, we performed confocal fluorescence microscopy (Fig. 3A). TPAP was mainly detected in the cytoplasm of pachytene spermatocytes and round spermatids, as previously reported [9]. GSG1 showed a scattered distribution in the cytoplasm of pachytene spermatocytes and round spermatids. In round spermatids, an extensive localization of GSG1 was also observed around the nucleus. Although TPAP and GSG1 existed in both pachytene spermatocytes and round spermatids, they were colocalized in the cytoplasm of round spermatids only. To better specify the intracellular localization of TPAP and GSG1, we examined cells at different stages of spermiogenesis (Fig. 3B). In early round spermatid, TPAP and GSG1 were colocalized in the vicinity of the nucleus. In case of cap-phase spermatid, interestingly, GSG1 showed its remarkable localization in the perinuclear region and it was more colocalized with TPAP in the cytoplasm than in that of early round spermatid. In elongating spermatid, however, GSG1 and TPAP scarcely showed their colocalization even though they existed.

3.4. Both TPAP and GSG1 localize in the endoplasmic reticulum (ER) of testicular cells

In Fig. 1B, GSG1 was separated as a membrane fraction in the cytoplasm of mouse testicular cells. To further define the subcellular localization of GSG1, we used confocal fluorescence microscopy. NIH/3T3 cells were transfected with GSG1-GFP. Unfortunately, the GSG1-GFP fluorescence signals were too weak. Consequently, we used GFP antibody and FITC-conjugated secondary antibodies to detect GSG1-GFP. GSG1 colocalized only with GRP78, an ER marker, suggesting localization in the ER (Fig. 4). Since the confocal microscopic data (Fig. 3) shows that TPAP colocalizes with GSG1 in the cytoplasm of spermiogenic cells, it is possible that TPAP interacts with GSG1 in the ER membrane. To test this theory, testicular extracts were subjected to a membrane flotation assay [16] by which the ER membrane can be separated from other cellular fractions. Our data show that both GSG1 and TPAP proteins exist in the ER membrane fraction of mouse testicular cells (Fig. 5). The quantitation of Fig 5B showed that TPAP in the ER membrane fraction constitutes about 8% of total cellular TPAP, suggesting that only part of TPAP is redistributed from the cytoplasm to the ER. The fact that all TPAP does not colocalize with GSG1 in the testicular cells supports this explanation (Fig. 3).

3.5. TPAP localizes to the ER via interactions with GSG1

To investigate the possible roles of GSG1 in ER localization of TPAP, we compared the subcellular distribution of TPAP in the absence and presence of GSG1. For this purpose, NIH/3T3 cells were transfected with FLAG-TPAP or cotransfected with both FLAG-TPAP and GSG1-GFP. In the absence of GSG1, the majority of FLAG-TPAP proteins dispersed throughout the cytoplasm, while upon addition of GSG1, FLAG-TPAP localized in the ER (Fig. 6). This finding suggests that GSG1 causes redistribution of TPAP from the cytosol to the ER.

4. Discussion

We identified GSG1 as a protein interacting with TPAP, using a yeast two-hybrid assay. Our data show that GSG1 localizes in the ER membrane. The putative transmembrane domains of GSG1 appear responsible for its ER localization. Notably, the expression of GSG1 and TPAP coincide during mouse spermatogenesis [10,13]. GSG1 interacts strongly with the N-terminal catalytic domain of TPAP in NIH3T3 cells. TPAP and GSG1 colocalize in round spermatid cells, especially in cap-phase spermatid cells. However, the elongating

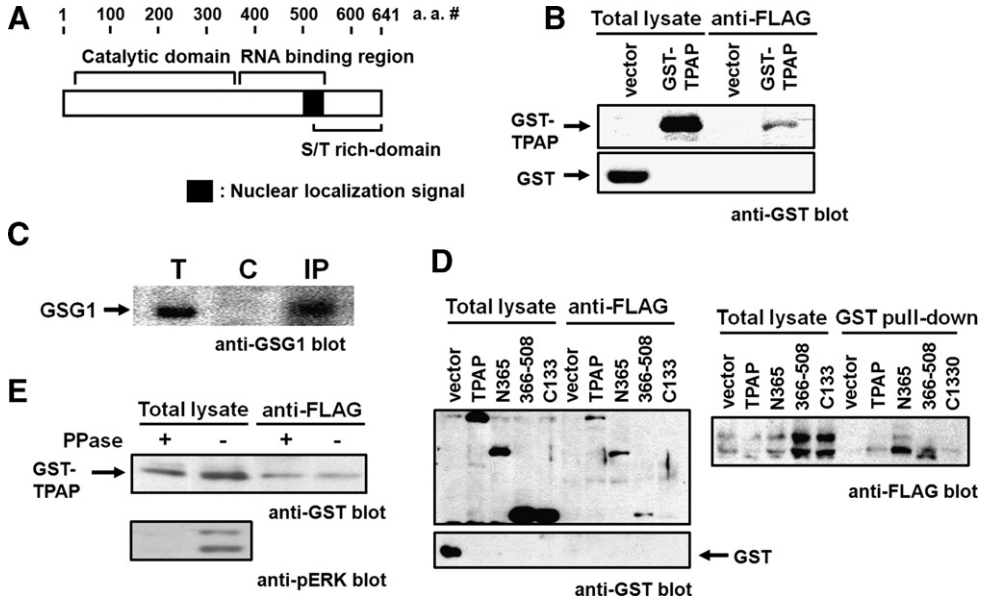

Fig. 2. In vivo interactions of TPAP with GSG1. (A) Schematic diagram presenting the significant features of TPAP. (B) Coimmunoprecipitation of GST-TPAP with FLAG-GSG1. Cell lysates from NIH/3T3 cells cotransfected with GST-TPAP and FLAG-GSG1 were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were subjected to immunoblotting using an anti-GST antibody. For the positive control, 1% of total lysates were used. (C) Interactions between endogenous TPAP and GSG1 in vivo. Coimmunoprecipitation was performed using testicular lysates obtained from 6-week-old mice. T, total lysate (1% input); C, control antibody; IP, immunoprecipitates with anti-TPAP. (D) Binding regions of TPAP for GSG1. Cell lysates from NIH/3T3 cells cotransfected with GST-TPAP derivatives and FLAG-GSG1 were subjected to coimmunoprecipitation assays. N365, catalytic domain (1–365); 366–508, RNA-binding domain; C133, S/T rich-domain (509–641). Left, total lysates (1% input) or their immunoprecipitates with anti-FLAG antibody were blotted with anti-GST antibody. Right, total lysates (1% input) or bound proteins on glutathione-sepharose beads (GST pull-down) were blotted with an anti-FLAG antibody. (E) Effect of dephosphorylation on interactions of TPAP and GSG1. Prior to coimmunoprecipitation assays, NIH/3T3 cell lysates cotransfected with GST-TPAP and FLAG-GSG1 were treated with λ phosphatase. A plus sign (+) represents phosphatase (PPase) treatment. Anti-pERK was used as a dephosphorylation control.

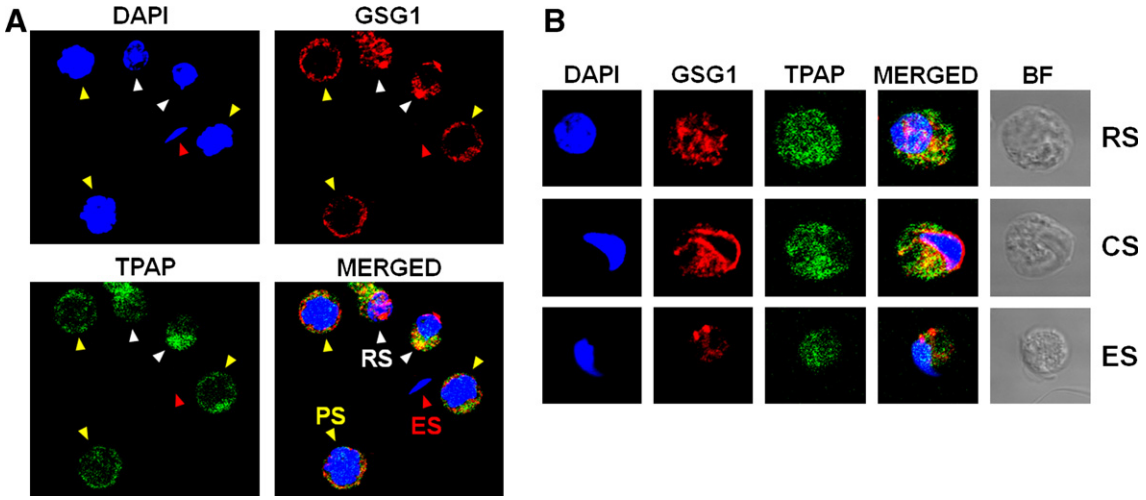

Fig. 3. Colocalization of GSG1 and TPAP in mouse spermatogenic cells. Fixed spermatogenic cells were incubated with anti-GSG1 and anti-TPAP followed by Cy3- and FITC-conjugated secondary antibody. GSG-1 and TPAP were visualized with a confocal fluorescence microscope. Nuclei were stained with DAPI (blue). (A) Overall localization of GSG1 and TPAP in mouse spermatogenetic cells. White, yellow, and red arrows represent pachytene spermatocytes, round spermatids, and elongating spermatid, respectively. (B) Localization of TPAP and GSG1 in cells at different stages of spermiogenesis. BF, bright field; RS, early round spermatid; CS, cap-phase spermatid; ES, elongating spermatid.

spermatid cells lack this colocalization. These results suggest that GSG1 interacts with TPAP at specific spermiogenic stages and that the interaction between TPAP and GSG1 would be related to the transformation of round spermatids to elongating spermatids.

We found that TPAP and GSG1 exist in the ER fraction of mouse testicular cells. Moreover, GSG1 expression affects the subcellular distribution of TPAP by targeting the cytosolic protein to the ER. In view of these findings, we propose that one biological function of GSG1 is to recruit TPAP to the

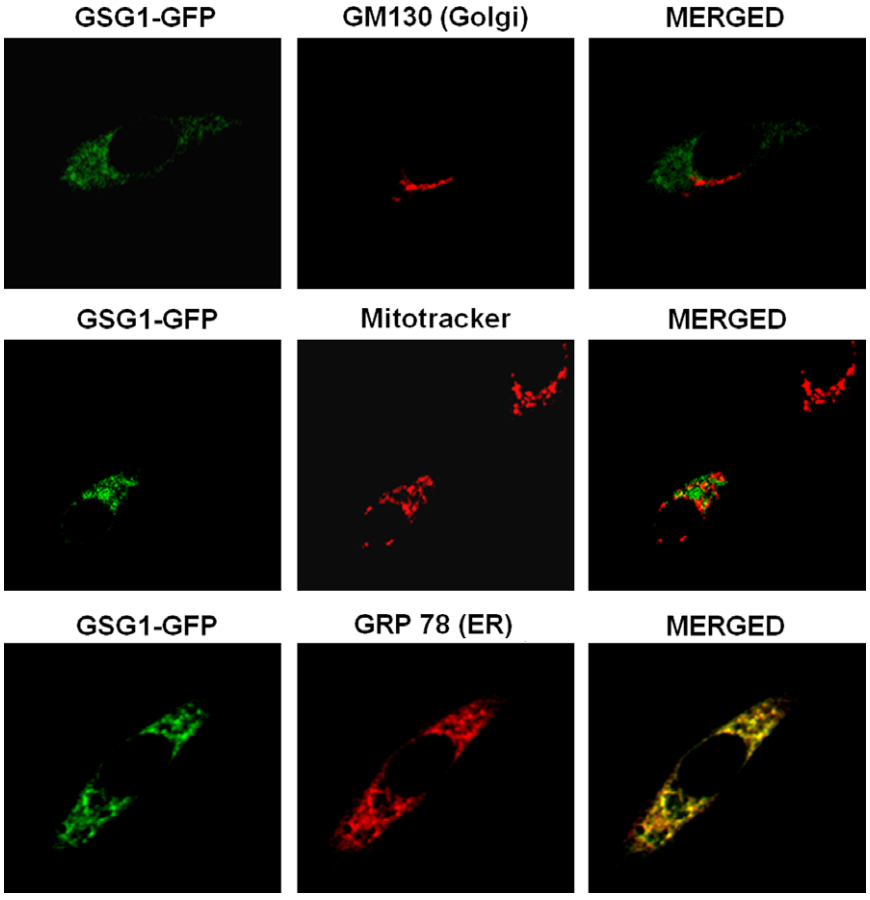

Fig. 4. Localization of GSG1 in NIH/3T3 cells. NIH/3T3 cells were transfected with GSG1-EGFP, and incubated with anti-GFP and anti-GM130 (Golgi marker) or anti-GRP 78 antibody (ER marker), followed by Cy3- or FITC-conjugated secondary antibody. For detection of mitochondria, transfected cells were incubated with Mitotracker (Invitrogen). Samples were visualized with a confocal fluorescence microscope.

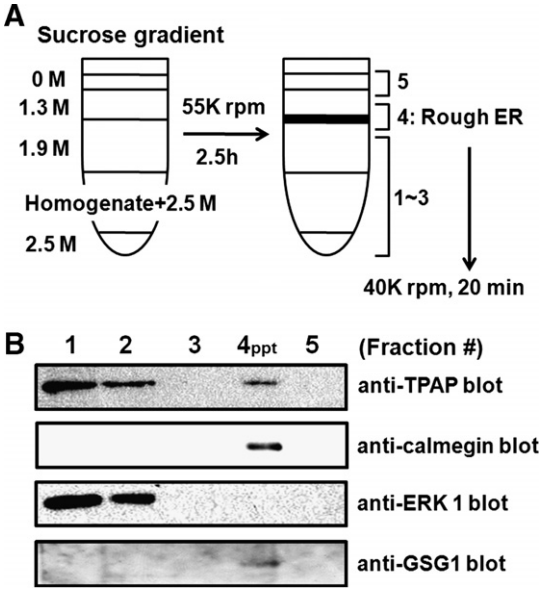

Fig. 5. Subcellular localization of GSG1 and TPAP in mouse testis. Testes from 6-week-old mice were subjected to membrane flotation assays [16]. (A) Each fraction is schematically shown. Fraction 4 was further centrifuged to obtain the ER fraction. (B) Each fraction was analyzed by immunoblotting. '4ppt' represents the ER fraction prepared from fraction 4 by the final centrifugation. Anti-ERK 1 and anti-calmegin antibodies were used as the cytosolic marker and testis-specific ER marker, respectively.

ER via protein–protein interactions. TPAP on the ER may preferentially add adenine residue at the 3′ ends of mRNAs for secretory/integral membrane proteins associated to ER-bound ribosomes. It has been known that ribosomes remain bound to the ER membrane following the termination of protein synthesis [17–19], and can initiate de novo protein synthesis without discriminating between mRNAs encoding soluble and signal sequence-bearing proteins [20]. In the unfolded protein response (UPR), transcripts of the key soluble stress proteins, XBP-1 and ATF-4, are translated primarily on ER-bound ribosomes [16]. In this respect, it is also possible that TPAP acts on mRNA to be associated with ER-bound ribosomes to generate soluble proteins.

The mechanisms and functions of TPAP-dependent cytoplasmic polyadenylation are yet to be elucidated. The mRNAs of particular transcription factors, such as TAF10, TAF12, and TAF13, are deadenylated in round spermatids of TPAP-deficient mice [11]. However, this does not affect their stability and translation. Interestingly, TAF10 is transported to the nucleus insufficiently in TPAP-deficient mice [11]. This result suggests that TPAP is involved in regulating the nuclear transport of TAF10, possibly via modulating the expression of transporter proteins through mRNA polyadenylation on the ER.

We show that TPAP and GSG1 are colocalized during spermiogenesis. Since the spermiogenic arrest occurs in TPAP-deficient mice [11], the interaction between TPAP and GSG1 may be related to the morphological change in spermiogenesis.

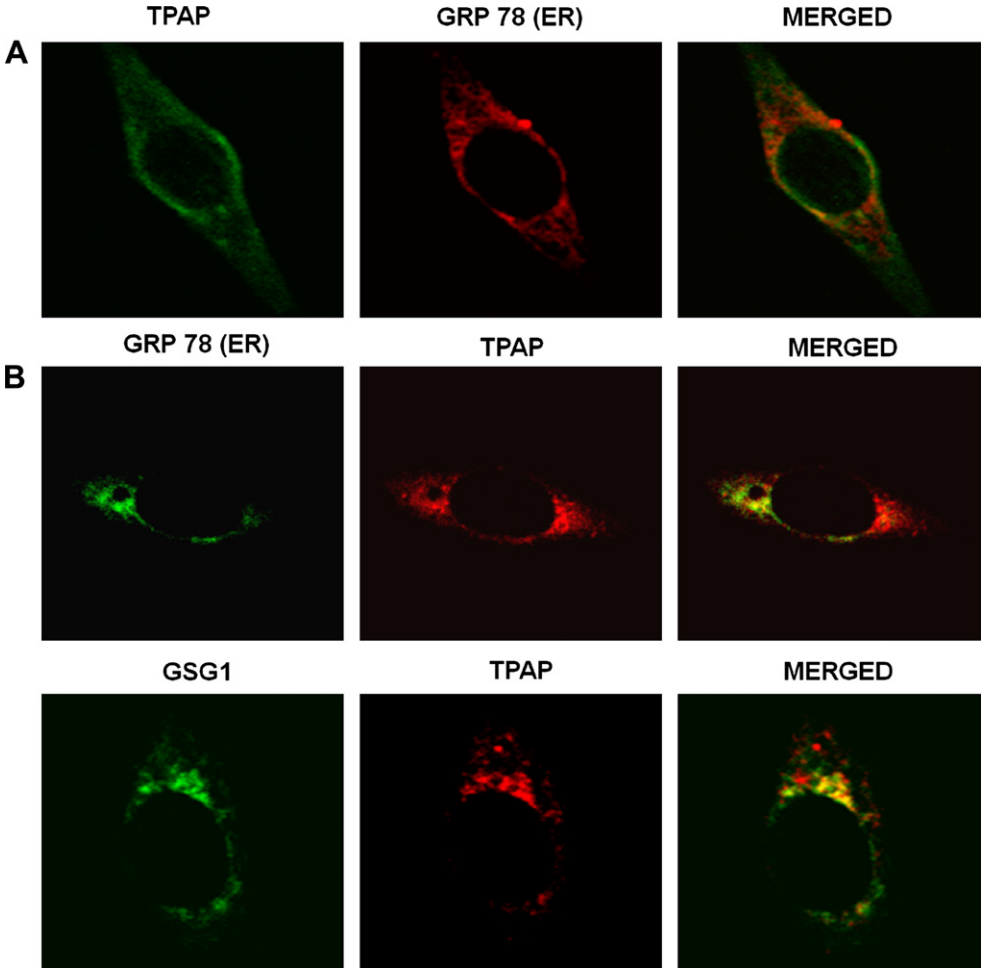

Fig. 6. Effects of GSG1 on the cellular localization of TPAP. NIH/3T3 cells were transfected with FLAG-TPAP alone (A) or both FLAG-TPAP and GSG1-GFP (B). Transfected cells were incubated with anti-FLAG (mouse), anti-GRP 78 (goat) and anti-GFP (rabbit) antibodies, followed by Cy3- or FITC-conjugated secondary antibody.

However, the real function of this interaction remains to be demonstrated.

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