Characterization of gametogenetin 1 (GGN1) and its potential role in male fertility through the interaction with the ion channel regulator, cysteine-rich secretory protein 2 (CRISP2) in the sperm tail

Duangporn Jamsai1,3, Deborah M Bianco1, Stephanie J Smith1,3, Donna J Merriner1,3, Jennifer D Ly-Huynh3,4, Amy Herlihy1, Birunthi Niranjan2, Gerard M Gibbs1 and Moira K O’Bryan1,3

1The Centre for Reproduction and Development and 2The Centre for Urological Research, Monash Institute of Medical Research, Monash University, 27-31 Wright Street, Clayton, Melbourne, Victoria 3168, Australia, 3The Australian Research Council (ARC) Centre of Excellence in Biotechnology and Development and 4Nuclear Signaling Laboratory, Department of Biochemistry and Molecular Biology, Monash University, Wellington Road, Clayton, Melbourne, Victoria 3800, Australia

Correspondence should be addressed to D Jamsai; Email: duangporn.jamsai@med.monash.edu.au

D Jamsai and D M Bianco contributed equally to this work

Abstract

Cysteine-rich secretory protein 2 (CRISP2) is a testis-enriched protein localized to the sperm acrosome and tail. CRISP2 has been proposed to play a critical role in spermatogenesis and male fertility, although the precise function(s) of CRISP2 remains to be determined. Recent data have shown that the CRISP domain of the mouse CRISP2 has the ability to regulate Ca\textsuperscript{2+} flow through ryanodine receptors (RyR) and to bind to MAP kinase kinase kinase 11 (MAP3K11). To further define the biochemical pathways within which CRISP2 is involved, we screened an adult mouse testis cDNA library using a yeast two-hybrid assay to identify CRISP2 interacting partners. One of the most frequently identified CRISP2-binding proteins was gametogenetin 1 (GGN1). Interactions occur between the ion channel regulatory region within the CRISP2 CRISP domain and the carboxyl-most 158 amino acids of GGN1. CRISP2 does not bind to the GGN2 or GGN3 isoforms. Furthermore, we showed that Ggn1 is a testis-enriched mRNA and the protein first appeared in late pachytene spermatocytes and was up-regulated in round spermatids before being incorporated into the principal piece of the sperm tail where it co-localized with CRISP2. These data along with data on RyR and MAP3K11 binding define the CRISP2 CRISP domain as a protein interaction motif and suggest a role for the GGN1–CRISP2 complex in sperm tail development and/or motility.


Introduction

Cysteine-rich secretory protein 2 (CRISP2), previously known as testis specific protein 1 (TPX1), is a testis-enriched protein that is incorporated into the acrosome of the sperm head and the sperm tail, specifically the outer dense fibers in the mid- and principal pieces, the longitudinal columns of the fibrous sheath in the principal piece, and the connecting piece that joins the sperm head to the tail (Hardy et al. 1988, Foster & Gerton 1996, O’Bryan et al. 2001, Busso et al. 2005). CRISP2 is a member of the CRISP family within the CRISP/antigen 5/pathogenesis related (CAP) superfamily of proteins. Within mammals, CRISPs show a strong expression bias to male reproductive tissues including the testis, prostate, and epididymis (Kohane et al. 1980, Roberts et al. 2002) and has been implicated as a decapacitation factor (Roberts et al. 2003, Nixon et al. 2006). CRISP3 is highly expressed in the epididymis, but also in the salivary glands, pancreas, B lymphocytes, and cancerous prostate (Kosari et al. 2002, Udby et al. 2002, 2005, Liao et al. 2003). CRISP4 expression is largely restricted to the epididymis and also been shown to bind to the sperm plasma membrane during epididymal maturation (Jalkanen et al. 2005, Nolan et al. 2006). The necessity for such a high concentration and diversity of CRISPs in relation to sperm biology remains unknown.

Structurally CRISPs are composed of two domains: an evolutionarily conserved N-terminal domain referred to as the CAP (Pr-1 or SCP) domain that is common to all CAP superfamily members and is ~20 kDa, and a unique C-terminal domain referred to as the CRISP domain that is ~6 kDa. The function of the CAP domain

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remains poorly defined, although there is evidence of it acting as a protease in cone snails and Xenopus (Milne et al. 2003, Schambony et al. 2003). CRISPs contain 16 absolutely conserved cysteine residues, 10 of which are concentrated in the CRISP domain. All cysteines are involved in intramolecular disulfide bonding to form two domain proteins separated by a hinge region (Eberspacher et al. 1995, Guo et al. 2005, Shikamoto et al. 2005, Wang et al. 2005). The CRISP domain of all reported CRISPs, however, may be further structurally divided into two subregions: the hinge region and the ion channel regulatory (ICR) region (Gibbs & O’Bryan 2007).

Based on mRNA expression and protein localization patterns, CRISP2 is proposed to have an essential role in spermatogenesis. CRISP2 has been implicated in the adhesion of transfected Jurkat cells into cultured Sertoli cells (Maeda et al. 1998, 1999) and when added in vitro has been found to interfere with sperm–oocyte binding (Busso et al. 2005). Although a sequence within the amino-terminal 101 amino acids of CRISP2 has been shown to facilitate cell–cell adhesion (Maeda et al. 1999), the context of expression, i.e., on the surface of a somatic cultured cell as opposed to intracellular organelles in haploid germ cells, and the protein confirmation, i.e., a linear peptide derived from a non-surface accessible portion of the protein in crystal structures (Busso et al. 2005, Guo et al. 2005), raise questions about the mechanisms of this action in vivo. Antibody-binding experiments do, however, suggest that a CRISP is involved in sperm–oocyte binding (Busso et al. 2005, 2007a, 2007b).

Recently, we have demonstrated that the CRISP domain of mouse CRISP2 can differentially regulate Ca$^{2+}$ influx through ryanodine receptors (RyR; Gibbs et al. 2006). This coupled with the localization of RyR to the connecting piece, and to a lesser extent the acrosome of human sperm (Harper et al. 2004), raises the possibility that CRISP2 plays a role in sperm motility and/or the acrosomal reaction. Furthermore, we have recently shown that the CRISP domain of mouse CRISP2 interacts with MAP kinase kinase kinase 11 (MAP3K11; Gibbs et al. 2007). MAP3K11 is a kinase with a role in activating the Jun NH$_2$-terminal kinase signaling (Chang & Karin 2001, Huang & Zhang 2003, Huynh et al. 2003) or in phosphorylating non-signal transduction proteins as exemplified by Golgi-associated protein, golgin-60, in somatic cells (Cha et al. 2004). CRISP2 and MAP3K11 are co-localized within the mouse sperm acrosome (Gibbs et al. 2007).

In order to further elucidate the biochemistry surrounding CRISP2 in spermatogenesis, the putative mature form of CRISP2 was used as bait in a yeast two-hybrid screening of an adult mouse testis cDNA library to identify interacting partners. Using this approach, several additional putative CRISP2 interacting proteins were identified, including gametogenetin 1 (GGN1). Ggn has more than ten splice variants giving rise to three proteins, GGN1, GGN2, and GGN3, with different subcellular localizations following transfection into HeLa cells (Lu & Bishop 2003). GGN1 (accession number AAP31497) is a 675 amino acid protein with no homologous functional motifs. The GGN3 isoform (accession number AAP31499) contains the carboxyl-most 137 amino acids of GGN1. GGN2 isoform (accession number AAP31498) shares the NH$_2$-terminal 205 amino acids in common with GGN1 after which a splicing-induced frame shift causes a complete divergence of protein sequences and a truncation at amino acid 271 (Lu & Bishop 2003). GGN1 and GGN3 were previously described as the binding partners of Fanconi anemia complementation group L (FANCL), originally known as proliferation of germ cells (Lu & Bishop 2003). FANCL is a ubiquitin E3 ligase and a component of the Fanconi anemia, which when mutated, such as in the gcd mouse, results in decreased primordial germ cell numbers and adult infertility (Agoulnik et al. 2002, Meetei et al. 2003). GGN1 was recently described as a binding partner of a novel protein, GGN-binding protein 1 (GGNBP1), and two previously characterized proteins, GGNBP2 (previously known as dioxin inducible factor-3, DIF-3) and ornithine decarboxylase antizyme 3 (OAZ3; Zhang et al. 2005, Zhao et al. 2005). The functional significance of these interactions is currently unknown. Despite convincing in vitro binding and mRNA expression data, the localization of GGN1 protein during spermatogenesis has not been reported previously, and as such it has remained difficult to predict its in vivo function.

In this study, we identify and characterize GGN1, a novel CRISP2-binding protein, in the mouse sperm tail. We demonstrate that the CRISP domain of CRISP2 binds to the carboxyl end of GGN1 in the yeast two-hybrid assay and we confirm their interaction using co-immunoprecipitations from the mouse testis tissue. Furthermore, we show that GGN1 protein is predominantly expressed during spermiogenesis and is incorporated into the principal piece of the sperm tail.

**Results**

**The ICR of CRISP2 binds to the carboxyl terminus of GGN1**

Using the putative mature form of mouse CRISP2 as bait in yeast two-hybrid screening, we identified GGN1 as a CRISP2-binding partner. Sequence alignment of all ten Ggn clones obtained in this screen revealed that they contained the 3’ region of the Ggn1 transcript. The shortest Ggn clone encoded the carboxyl-most 158 amino acids of the Ggn1 transcript (as typified by clone B13; Fig. 1A). The sequence of the B13 clone appeared to overlap completely with both Ggn1 and Ggn3. However, closer scrutiny revealed that all clones either extended 5’ or 3’ of the Ggn3, clearly defining them as being Ggn1.

To determine which part of Ggn1 mediated the interaction with CRISP2 and to further assess the potential for the Ggn3 isoform to interact, two additional Ggn
constructs were generated. GgnΔ1 was designed to contain the region unique to the GGN1 isoform (amino acid positions 206–538) and GgnΔ3 was designed to contain the coding region of the predicted GGN3 protein (amino acid positions 539–675 of GGN1; Fig. 1A). Constructs were co-transfected with mature Crisp2 and Crisp2Δ5 bait vectors. Neither of the Ggn deletion constructs interacted with mature Crisp2 or the ICR region of CRISP2. These data clearly define that CRISP2 interacts with amino acids 518–675 of GGN1 but that it does not bind to the GGN3 isoform.

To define which regions of CRISP2 interact with GGN1, seven Crisp2 deletion constructs were generated (Fig. 1B) and co-transfected with Ggn clone B13 (amino acid positions 518–675) into yeast. As shown in Fig. 1B, only those clones containing the ICR region of the CRISP domain survived leucine–tryptophan–histidine dropout selection. The interaction did not appear to rely on the presence of any part of the CAP domain or the hinge region.

**Ggn is a testis-enriched transcript**

Ggn mRNA species were detected by northern blotting only in the developing adult testis and epididymis (Fig. 2). Using a probe that would theoretically bind to all three Ggn isoforms, a predominant 1.9 kb band and a less intense 5 kb band were detected. The 1.9 kb band is consistent with the Ggn1 isoform (Lu & Bishop 2003). However, the identification of the 5 kb band is unknown and has not been reported previously. The onset of Ggn1 expression within the developing testis was ~ 18 days post partum consistent with expression in late pachytene spermatocytes. The level of expression was up-regulated through to 36 days post partum, suggesting that Ggn1 is also expressed in round and elongating spermatids.

Although we showed that Ggn1 is a testis-enriched transcript and proposed its potential role in spermato genesis through the interaction with CRISP2, a low level of Ggn expression in ovary detected by RT-PCR analysis has been reported previously (Lu & Bishop 2003). A close examination of GEO profiles reveals that Ggn is also expressed in ovulated oocytes and in the early-stage embryo. These data suggest a potential role for Ggn in embryo development and/or implantation. In addition, UniGene EST database and our RT-PCR analysis (data not shown) suggest that Ggn is also expressed in skeletal muscle. Due to the fact that Ggn has multiple splice variants that can give rise to at least three protein isoforms, it is possible that different forms of GGN could be preferentially expressed in different tissue types, although it is not clear at this stage which GGN isoform(s) is found in ovary, ovulated oocyte, or skeletal muscle. Further investigation and functional analysis of the consequences of altered Ggn function in in vivo animal models remain to be addressed.
**GGN1 is a 69–71 kDa protein**

Recently, it has become apparent that three mouse Ggn1 mRNA sequences have been reported on public databases as accession numbers AF538032, BC089358, and NM_182694. These sequences encode a 675 amino acid protein (predicted MW 69 kDa), a 673 amino acid protein (predicted MW 68.9 kDa), and a 709 amino acid protein (predicted MW 71 kDa) respectively (Fig. 3A). The 675 amino acid form contains an insertion of two alanine residues compared with the 673 and 709 amino acid forms. In addition, the 709 amino acid form contains an extra 36 amino acids inserted prior to the ATG codon of the 673 amino acid form while the rest of the sequence is identical to the 673 amino acid form due to the use of an alternate C-terminal ATG start codon (Fig. 3A). Based on the available information from public databases, the AF538032 mRNA sequence encoding the 675 amino acid form was obtained from BALB/c mouse strain while the other two sequences were obtained from C57BL/6 strain. To further investigate the difference among the three reported forms of Ggn1, we performed genomic DNA sequencing on three different mouse strains including C57BL/6, BALB/c, and CBA. Our data confirmed that the six nucleotides (CGGCGG) in-frame insertion resulting in the extra two alanine residues in the 675 amino acid form was present in genomic DNA from BALB/c mice but was not present in genomic DNAs from the CBA and C57BL/6 mice (Fig. 3B). These data indicate that the small sequence variations observed in GGN1 protein are a result of genetic background variations.

To assess the presence and molecular mass of GGN1 protein in the mouse testis and sperm, we performed Western blot analysis using a GGN1-specific peptide antibody that would detect all three GGN1 forms. As shown in Fig. 3C, a predominant band of ~69 kDa corresponding to the 675 and 673 amino acid forms was seen in both the testis and epididymal sperm of F1 hybrid mice (CBA×C57BL/6). A slightly larger band (~70–71 kDa) was also detected in the testis extract, but was not detected in the sperm. All bands disappeared if the antisemur was pre-absorbed with the immunizing peptide (Fig. 3D), thus indicating the specificity of the method.

**In vitro interaction of CRISP2 and GGN1**

Co-immunoprecipitation was used to further confirm the in vitro interaction between CRISP2 and GGN1 (Fig. 4). The monoclonal CRISP2 antibody was used to immunoprecipitate the CRISP2–GGN1 complex from an adult mouse testis lysate. As shown in Fig. 4, GGN1 and CRISP2 proteins were only detected from the immune complexes precipitated with the CRISP2 antibody. Negative control experiments using no antibody and an irrelevant mouse IgG antibody showed neither a CRISP2 nor a GGN1 band, indicating the specificity of the CRISP2–GGN1 interaction observed from co-immunoprecipitation.

**GGN1 is localized to the mouse sperm tail**

The localization of GGN1 protein has not previously been reported and as such the biological context within which the CRISP2–GGN1 interaction has a role was unknown. GGN1 protein localization within the adult mouse testis was determined by immunohistochemistry (Fig. 5A–C). Consistent with the northern blotting data, GGN1 protein first appeared during spermatogenesis in the cytoplasm of late pachytene spermatocytes (Fig. 5B) and was up-regulated in round spermatids before being incorporated into the growing sperm tail (Fig. 5C). These data show that unlike Crisp2, Ggn1 does not appear to undergo a translational delay. Previously we, and others (O’Bryan et al. 1998,

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**Figure 3** GGN1 is a 69–71 kDa protein detected in both testis and sperm. (A) Sequence alignment of three reported GGN1 protein sequences. The remainder of the N-terminal sequences is 100% identical among three sequences (not shown). (B) Ggn genomic DNA sequencing data alignment from BALB/c, C57BL/6, and CBA strains. (C) Western blot of mouse testis and sperm extracts using GGN1-p1 antibody. (D) Western blot of mouse testis and sperm extracts using GGN1-p1 antibody pre-absorbed with 50-fold molar mass excess of immunizing peptide.
2001, Maeda et al. 1999), have shown the Crisp2 mRNA is also first expressed in late pachytene spermatocytes, and following a translational delay is expressed in round spermatids through to elongated spermatids where it is incorporated into the developing acrosome and sperm tail.

To assess the potential for CRISP2 and GGN1 co-localization, co-immunofluorescence of mouse sperm was also performed. GGN1 (Fig. 5D–F) and CRISP2 (Fig. 5G) were co-localized to the principal piece of the sperm tail (Fig. 5H). These data suggest that GGN1 and CRISP2 are co-localized to the same region in the sperm tail and support the possibility of their interaction in vivo.

Discussion

CRISP2 and other CRISPs have been proposed to play a role in male germ cell development and function; however, the lack of in vivo animal models and knowledge on biological pathway(s) in which CRISPs are involved has limited the identification of their precise function. In an attempt to define the role of CRISP2, we carried out a screen for CRISP2 interaction partners during spermatogenesis by way of a yeast two-hybrid system. Several putative CRISP2-binding proteins were identified including MAP3K11 (Gibbs et al. 2007). In this study, we report for the first time that GGN1 is a novel CRISP2-binding partner during spermatogenesis. Our northern blot data generated with the first wave of spermatogenesis and previous in situ hybridization (Lu & Bishop 2003) indicated that Ggn1 mRNA is first expressed in late pachytene spermatocytes and is up-regulated in round spermatids. In agreement, GGN1 protein localization as determined by immunohistochemistry on established spermatogenesis confirmed that GGN1 protein is also first appears in late pachytene spermatocytes and co-localizes with CRISP2 in spermatids before being incorporated into the principal piece of the sperm tail. These data suggest that the CRISP2–GGN1 complex plays a role of the sperm tail assembly and/or motility.

Using GGN1 antibody in Western blot analysis, a 69 kDa band was detected in both testis and sperm extracts. A larger band of about 70–71 kDa also appeared in the testis but was not observed in the sperm, indicating that this form of protein is either not incorporated into sperm tails or it undergoes post-translational modifications after being incorporated into sperm tails. In addition, a 69 kDa band in the sperm extract appeared to be a doublet with a slightly

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**Figure 4** CRISP2 interacts with GGN1 in vitro as determined by co-immunoprecipitation from the mouse testis extracts. The CRISP2–GGN1 complexes in the mouse testis extract were immunoprecipitated with the monoclonal CRISP2 antibody. Negative controls; no antibody added and a mouse IgG antibody were included to confirm the specificity of the method.

**Figure 5** GGN1 protein first appears in late pachytene spermatocytes and spermatids and incorporates into the sperm tail. Localization of GGN1 protein in the testis was determined using immunohistochemistry on wild-type mouse testis sections (A–C). No positive immunoreactivity was observed in the antibody pre-absorption control (A). GGN1 protein was detected in the cytoplasm of late pachytene spermatocytes and was up-regulated in round spermatids before being incorporated into the growing sperm tails (B–C). EP, early pachytene spermatocyte; MP, mid-pachytene spermatocyte; LP, late pachytene spermatocyte; R, round spermatid; E, elongated spermatid; T, sperm tail. Scale bars represent 50 mm. Stages of seminiferous tubules are shown in the top right corners. Localization of GGN1 in caudal mouse sperm was determined by immunofluorescence (D–E). Sperm chromatin was visualized using DAPI and immunostaining of GGN1 protein was specific to only the principal piece of sperm tail as shown in bright field image (E). The bright field image has been artificially colored to enhance clarity. Scale bars represent 100 μm. Co-localization of GGN1 and CRISP2 in the mouse sperm was determined by co-immunofluorescence. GGN1 localization (D–F); CRISP2 localization (G); GGN1 and CRISP2 co-localization (H, yellow signal indicates CRISP2–GGN1 co-localization).
larger sized band. This larger band could possibly be a post-translationally modified form of the 69 kDa band and/or the 70–71 kDa band presenting in the testis extract. Further investigations are required to address this point.

Based on the current available data on public databases, GGNs share no primary sequence homology to proteins of known function and possess no homologous motifs and as such it has remained difficult to predict their in vivo function(s). GGN1, the longest isoform, contains a characteristic proline-rich C-terminal portion. Proline-rich regions have been proposed to play a role in protein–protein interactions in various protein complexes (Kay et al. 2000). Using deletion studies in the yeast two-hybrid assay, we demonstrated that GGN1 binds to CRISP2 through its carboxyl-most 158 amino acids and interacts with the ICR of the CRISP domain of CRISP2. It is noted that 17 out of 135 proline residues are located within this region of the GGN1 isoform. We have also shown that GGN3, the shortest isoform that contains the carboxyl-most 137 amino acids of GGN1, does not bind to CRISP2. Furthermore, no clones encoding the GGN2 isoform were identified from our screen. Sequence comparison of clone B13 (158 amino acids), the shortest clone identified as a CRISP2-binding partner, and GGN3 revealed that clone B13 contained 21 amino acids (ATPATVTSQVPATATAELSPP) upstream of the GGN3 isoform, suggesting that this region is important for CRISP2–GGN1 interaction. Further site-directed mutagenesis studies would greatly facilitate the identification of amino acid residues that are critical for GGN1–CRISP2 interaction. The interaction between GGN1 and CRISP2 was confirmed using co-immunoprecipitation from the mouse testis extract. A 69 kDa band corresponding to the GGN1 isoform was detected thus confirming the interaction data obtained from the yeast two-hybrid system. In addition to GGN1, we have previously shown that the CRISP domain of CRISP2 is responsible for binding to the carboxyl-most 20 amino acids of MAP3K11 within sperm acrosome. MAP3K11 is another example of a CRISP2-binding partner that also contains C-terminal SH3 domain containing proline-rich kinase (Gallo et al. 1994). Our result showed that both GGN1 and MAP3K11 interact with CRISP2 via the CRISP domain that we have shown can differentially regulate Ca$^{2+}$ gating through the ryanodine ion channel receptor (Gibbs et al. 2006). The regulated movement of Ca$^{2+}$ is essential for several aspects of sperm function including sperm capacitation or maturation within the female reproductive tract, the acrosome reaction, and sperm motility. Based on the expression and the co-localization of the GGN1–CRISP2 complex, we propose that GGN1 plays a role in sperm motility and/or capacitation through the interaction of CRISP2.

In conclusion, this study provides new insights toward the understanding of the expression and function of GGN1 and CRISP2. These data along with those showing that the CRISP domain of CRISP2 is responsible for interactions with the RyRs (Gibbs et al. 2006) and MAP3K11 (Gibbs et al. 2007) clearly define the CRISP2 CRISP domain as a protein interaction motif. Such an interaction raises the possibility of a role for GGN1–CRISP2 complex in sperm tail development or the regulation of the Ca$^{2+}$ flux associated with sperm motility or maturation in the female reproductive tract.

Materials and Methods

Yeast two-hybrid screening

The putative mature Crisp2 cDNA coding region (amino acids 23–243) was cloned into the NcoI and EcoRI sites of the pAS2-1 bait vector (Clontech) to make a fusion protein between the yeast GAL4 DNA-binding domain and CRISP2 as described previously (Gibbs et al. 2007). A mouse testis expression library was screened for CRISP2-binding proteins using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Crisp2/pAS2-1 bait vector was co-transfected with the prey vectors, mouse testis transcripts cloned into pACT2 prey vector, into the reporter Saccharomyces cerevisiae strain P69-4A. Cells were plated on nutrient-deficient media (leucine–tryptophan–histidine dropout) to screen for interacting partners according to the manufacturer’s instructions. Positive clones were subsequently cloned into pGEMTeasy using pACT2F: 5’-GGCCAGATTGAAACTTAGGG-3’ and pACT2R: 5’-ATACCCCAACACCCCAA-3’ primers prior to DNA sequencing.

Deletion studies to define regions of CRISP2–GGN1 interaction

Seven Crisp2 deletion constructs (Fig. 1B) were cloned into the pAS2-1 bait vector and two Ggn constructs (Fig. 1A) were cloned into the pACT2 prey vector using primers flanking the EcoRI and NcoI sites. Crisp2 deletion construct 1 (Crisp2Δ1, amino acids 79–243) was amino-terminally truncated to omit the putative cell adhesion domain (Maeda et al. 1999). Crisp2 deletion construct 2 (Crisp2Δ2, amino acids 23–188) was carboxy-terminally truncated to remove the CRISP domain. Crisp2 deletion construct 3 (Crisp2Δ3, amino acids 189–243) contained only the CRISP domain (composed of both the hinge and ICR regions). Crisp2 deletion construct 4 (Crisp2Δ4, amino acids 23–154 fused to amino acids 189–243) had the mid-region encoding part of the CAP signature motif removed and would remove three out of the six cysteine involved in intramolecular disulfide bonding within the CAP domain (Eberspaecher et al. 1995, Guo et al. 2005); Crisp2 deletion construct 5 (Crisp2Δ5, amino acids 203–243) contained the ICR of the CRISP domain. Crisp2 deletion construct 7 (Crisp2Δ7, amino acids 23–203) contained the mature Crisp2 coding with the ICR of the CRISP domain deleted. Crisp2 deletion construct 8 (Crisp2Δ8, amino acids 188–203) contained only the hinge region. Reference Ggn1 mRNA and GGN1 protein sequences were AF538032 and AAP31497 (675 amino acids) respectively. GgnΔ1 contained the region unique for GGN1 (amino acids 206–538) and GgnΔ3 contained the...
coding region of predicted GGN3 protein (amino acids 539–675 of GGN1). Prey and bait constructs were co-transfected and selected in yeast as outlined above to define the regions of CRISP2 and GGN, which interact.

**Northern blot analysis**

The developmental expression of Ggn within mouse testis and somatic tissues was determined by northern blotting as described previously (Hickox et al. 2002). Testis samples were harvested from ages 0, 14, 18, 22, 30, and 36 days after birth and experiments were conducted in accordance with the National Health and Medical Research Council’s Guidelines on Ethics in Animal Experimentation and were approved by the Monash Medical Centre Animal Experimentation Ethics Committee. Isolation of total RNA was performed as described previously (Gibbs et al. 2007). A 185 bp cDNA probe designed to hybridize to Ggn1, Ggn2, and Ggn3 mRNAs (Fig. 1A) was amplified from mouse testis total cDNA using primers XM145491-2F 5’-CGTGAGAGGAGGACTTG-3’ and XM145491-2R 5’-CAGTAGACCTTGCAGGG ACA-3’. The membrane used for Ggn probe hybridization had previously been probed with Map3k11 probe (Gibbs et al. 2007). In order to avoid stripping the membrane, the Map3k11-probe membrane was left for over 30 days to allow a total reduction of 32P radioactivity. The presence of residual radioactivity was assessed prior to re-probing with the Ggn probe.

**Antibody production and purification**

Anti-peptide sera were generated in goats by Antibodies Australia (Werribee/Clayton, VIC, Australia). Briefly, a peptide with 100% identity to mouse GGN1, termed p1 (amino acid positions 405–423, RSGQTHPSGPRPPTPAL; Fig. 1A) was synthesized (Mimotopes, Clayton, VIC, Australia) and coupled through an N-terminal cysteine to diphtheria toxoid (DT). DT-conjugated GGN1-p1 was used to immunize three goats. The immune response and antibody titer were monitored by ELISA. Following the immunization cycle, an antiserum to GGN1-p1 was purified using a peptide p1 affinity column. Peptide p1 was coupled to SulfoLink (Pierce, Rockford, IL, USA) agarose gel at 1 mg peptide/ml gel using the recommended protocol with one modification. To ensure all cysteines in the peptide were in the reduced state to maximize coupling efficiencies, the peptides were incubated for 30 min in 5 mM Tris(2-carboxyethyl)phosphine hydrochloride reductant (Sigma) and added directly to the SulfoLink coupling gel. Serum was diluted tenfold in PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl (pH 7.4)) containing protease inhibitors cocktail Set III (Calbiochem, San Diego, CA, USA) and passed through the GGN1-p1 column, washed with 12 column volumes of PBS buffer, and eluted with 8X1 ml fractions (8 column bed volumes) of 0.1 M glycine (pH 2.5) into an equal volume of 1 M Tris (pH 8.5) to neutralize the pH. Antibody elution was monitored by absorption at A280 and peak fractions were combined, dialyzed against PBS, and stored in 50% glycerol and 0.05% azide at 20°C. The specificity of the antiserum was determined by Western blotting of testis and caudal epididymal sperm extracts and through pre-absorption of the antiserum using a 50-fold molar excess of the peptide in immunochromical studies.

**Western blot analysis**

Mouse sperm were prepared by dissecting out the caudal epididymis and piecing the wall with a scalpel so as to allow sperm to swim out into pre-warmed PBS buffer at 37°C. Sperm were washed with 3×1 ml PBS and epididymal proteins were removed by centrifugation at 1000 g for 30 s. Sperm were resuspended in 1 ml Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 50 mM Tris, 1% NP-40 (pH 7.0)) and passed through a narrow gauge needle to shear DNA prior to electrophoresis. Mouse testis was homogenized in NP-40 lysis buffer containing protease inhibitors using a 0.1 µm Wheaton borosilicate glass境外 and particular material was removed by 2×5 min centrifugation at 18 000 g at 4°C. Soluble sperm and testis proteins were denatured at 90°C in reducing SDS-PAGE sample buffer prior to electrophoresis through a 10% polyacrylamide gel. Equal amounts of testis and sperm proteins (20 µg) were loaded onto the gel. The proteins were transferred to nitrocellulose membranes (GE Biosciences, Piscataway, NJ, USA) followed by blocking in 50% Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and 50% PBS. The membranes were incubated with the GGN1-p1 primary antibody (3.1 µg/ml in 50% Odyssey blocking buffer/50% PBS) overnight at 4°C with gentle mixing. Following 3×10 min washes with PBS containing 0.05% Tween 20, donkey anti-goat Alexa Fluor secondary antibody (Invitrogen, 0.2 µg/ml in 50% Odyssey blocking buffer/50% PBS) was added and incubated for 1 h at room temperature. Following 3×10 min washes with PBS buffer containing 0.05% Tween 20, the membranes were scanned using the Odyssey scanner. Pre-adsorption of the GGN1-p1 antibody with the GGN1-p1 immunopeptide was done with a 50-fold molar excess of peptide at 4°C overnight and subsequently used in Western blot as described above.

**Co-immunoprecipitation**

CRISP2 and GGN1 were co-immunoprecipitated from an adult mouse testis tissue using the Catch and Release v2.0 Reversible Immunoprecipitation System (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) according to the supplier’s instructions. Briefly, 100 µg mouse testis lysate, 10 µg monoclonal CRISP2 antibody (R&D Systems, Minneapolis, MN, USA), and 1.2 µg antibody capture affinity ligand were mixed in 1×Catch and Release wash buffer to a final volume of 500 µl in the Catch and Release spin columns. Immunoprecipitation was carried out for 16 h at 4°C on a rocking platform. The columns were washed three times with 400 µl 1×Catch and Release wash buffer and the immune complexes were eluted with 70 µl 1×, 2×, and 4× of the Catch and Release non-denaturing elution buffer followed by 70 µl Catch and Release denaturing elution buffer. Negative controls, mouse IgG (Invitrogen, 10 µg), and PBS were included. Equal amounts of the immune complexes and washed immunoprecipitates were separated by SDS-PAGE. For GGN1 detection, Western blots were conducted as described above except...
the donkey anti-goat IRDye 800CW secondary antibody (Odyssey, 0.066 μg/ml in 50% Odyssey blocking buffer/50% PBS; LI-COR Biosciences) was used. For CRISP2 detection, CRISP2 MAB (0.5 μg/ml in 50% Odyssey blocking buffer/50% PBS) and goat anti-mouse 680 Alexa Fluor secondary antibody (Invitrogen, 0.2 μg/ml in 50% Odyssey blocking buffer/50% PBS) were used.

**Immunohistochemistry**

Testis samples from adult mice (10 weeks old) were fixed in Bouin’s fixative solution and embedded in paraffin wax using standard methods. Five micrometer sections were cut and dewaxed prior to antigen retrieval by microwaving the sections in 10 mM citrate buffer (pH 6.0) for 16 min. Endogenous peroxidase activity was blocked with 3% H2O2 for 5 min and the sections were blocked in CAS Block (Zymed, South San Francisco, CA, USA). The proteins were detected by incubating the sections at 4°C overnight with the affinity purified goat anti- GGN-p1 primary antibody (0.8 μg/ml, in Dako antibody diluent; Dako Cytomation, Carpinteria, CA, USA). Unbound antibody was removed by washing with PBS buffer followed by incubation with biotinylated rabbit anti-goat secondary antibody (Zymed, 7.5 μg/ml in Dako antibody diluent) for 30 min. The primary and secondary conjugates were visualized by Streptavidin HRP (Millipore, Billerica, MA, USA; Cat. No. 988210020, 1:200 in Dako antibody diluent) for 30 min at room temperature and the peroxidase activity was detected using 3',3'-diaminobenzidine (Dako Liquid DAB). The sections were counterstained with Harris’ hematoxylin, dehydrated, and mounted in DPX. Antibody pre-adsorption control was performed using 50-fold molar excess of GGN1-p1 peptide at 4°C for 6 h before being applied to the sections in place of primary antibody alone.

**Immunofluorescence**

Caudal mouse sperm from adult mice were collected as described above. Sperm were passed through a 100 μm mesh filter and spotted onto Superfrost plus slides (Microim International, Walldorf, Germany) and dried on a warm plate. Sperm were fixed for 2 min in methanol: acetone (1:1 v/v) followed by PBS washing. Permeabilization was performed in 0.2% Triton X-100 (in 10% normal horse serum in PBS) for 30 min followed by washing with PBS buffer and blocking in 10% normal horse serum in CAS Block for 30 min. The slides were incubated with goat GGN-p1 primary antibody (6.2 μg/ml) at 4°C overnight. Unbound antibody was removed with PBS buffer prior to incubation with donkey anti-goat secondary antibody (Alexa Fluor 488, 10 μg/ml) for 2 h at room temperature. Cells were counterstained with DAPI and mounted with Dako fluorescent mounting media.

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759


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