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0610009K11Rik, a testis-specific and germ cell nuclear receptor-interacting protein

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Abstract

Using an *in silico* approach, a putative nuclear receptor-interacting protein 0610009K11Rik was identified in mouse testis. We named this gene as testis-specific nuclear receptor-interacting protein-1 *(Tnrip-1)*. *Tnrip-1* was predominantly expressed in the testis of adult mouse tissues. Expression of *Tnrip-1* in the testis was regulated during postnatal development, with robust expression in 14-day-old or older testes. *In situ* hybridization analyses showed that *Tnrip-1* is highly expressed in pachytene spermatocytes and spermatids. Consistent with its mRNA expression, Tnrip-1 protein was detected in adult mouse testes. Immunohistochemical studies showed that Tnrip-1 is a nuclear protein and mainly expressed in pachytene spermatocytes and round spermatids. Moreover, co-immunoprecipitation analyses showed that endogenous Tnrip-1 protein can interact with germ cell nuclear receptor (GCNF) in adult mouse testes. Our results suggest that Tnrip-1 is a testis-specific and GCNF-interacting protein which may be involved in modulation of GCNF-mediated gene transcription in spermatogenic cells within the testis.

Keywords

spermatocyte; spermatids; GCNF; nuclear receptor; testis; mouse; in silico; protein-protein interaction

Introduction

Spermatogenesis is the process that occurs in the seminiferous tubules of the testis leading to the production of mature sperm [1]. This process includes the spermatogonial phase, which involves the proliferation of cells via mitosis, the spermatocyte phase, in which recombination and meiosis occurs to yield haploid cells, and the spermatid stage in which terminal differentiation of the haploid spermatids yields mature sperm [1]. This complex process is at least partially controlled by transcriptional factors such as nuclear receptors within spermatogenic cells. Germ cell nuclear factor (GCNF) is a member of the nuclear receptor superfamily [2;3;4] and plays a critical role in normal embryonic development and embryonic stem cell differentiation [5;6]. It can bind to a direct repeat (DR) of the AGGTCA sequence (DR0) and repress gene transcription likely by recruiting other proteins such as nuclear receptor co-repressors 1 and 2 (Ncor-1 and Ncor-2), and ubiquitin interaction motif containing 1 (RAP80) [7;8;9;10;11;12]. In adult vertebrates, *GCNF* is predominantly expressed in the testis and ovary of all species analyzed, including mouse, rat, and human [13;14;15]. In the testis,

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GCNF is predominantly expressed in round spermatids in rodents [2;3;13;14;16] and pachytene spermatocytes in human [17]. Previous studies have shown that GCNF can bind to the DR0 elements in the promoters of mouse *protamine 1* and *2* genes, which are required for normal spermatogenesis and male fertility [18;19], and repress their activities in cultured cells [9;20; 21]. In addition, we observed that *GCNF* mutant mice with a mosaic deletion of *GCNF* in spermatogenic cells are subfertile (Lan, Xu and Cooney, unpublished data). Thus, GCNF will likely play a role in the regulation of gene transcription within spermatogenic cells, affecting male fertility.

In general, nuclear receptors can dynamically interact with coactivators or corepressors in the presence or absence of their cognate ligands to regulate gene transcriptions in target cells to elicit their biological functions [22;23;24]. Identification of novel GCNF-interacting proteins in spermatogenic cells will lead to a better understanding of the action of GCNF during normal spermatogenesis. In this report, we identified a really interesting novel gene (RING) *0610009K11Rik* using an *in silico* approach. We named this gene as testis-specific nuclear receptor-interacting protein-1 *(Tnrip-1)*. Expression analyses showed that *Tnrip-1* is a testis-specific factor and predominantly expressed in pachytene spermatocytes and round spermatids at both mRNA and protein levels. Moreover, we found that Tnrip-1 protein can interact with GCNF protein in mouse testes. Our results suggest that Tnrip-1 is a testis-specific and GCNF-interacting protein that is predominantly expressed in spermatogenic cells within the testis.

Materials and methods

In silico analysis

Mouse cDNA sequences enriched in testis were obtained using the cDNA Digital Gene Expression Displayer (DGED) program (http://cgap.nci.nih.gov/Tissues/GXS), similar to a previous report [25]. Expressions of these genes in the testis were then validated from multi-tissue mouse microarray data generated from the Genomics Institute of the Novartis Research Foundation (http://symaltas.gnf.org) [26]. These testis-enriched cDNAs were then subjected to computer analysis to identify proteins with an Ile/Leu xx Ile/Val Ile (I/LXXI/VI) motif, which is also called the CoRNR box [27;28].

Animal tissue preparations

Adult C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and maintained on a 14 hour light:10 hour dark cycle, with free access to food and water, in the vivarium of the University of Louisville. Animals were euthanized with carbon dioxide and tissues were trimmed free of fat and then subjected to homogenization, RNA isolation or fixation. Whole-cell homogenates from adult mouse tissues were prepared as described previously [29]. For *in situ* hybridization studies, adult testes were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin-embedded tissues were sectioned at 7-μm thickness with a Richard-Allan Scientific MICROM HM325 microtome (Fisher Scientific, Pittsburgh, PA). For immunohistochemical studies, adult testes were frozen in Tissue-Tek O.C.T. compound (VWR International, Inc, Batavia, IL) and sectioned at 5-μm thickness with a Leica-CM1900 cryostat (Leica Mircrosystems Inc., Bannockburn, IL). All experiments in this study were approved by the Animal Welfare Committee of the University of Louisville.

RNA analyses

Total RNA from testes and other tissues was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Semi-quantitative RT-PCR analyses of *Tnrip-1* were performed using PCR primers (5′-GCCGTACTGTACTCCATATACC-3′ and 5′-TTCAGCATCTCCTCTGTCTC-3′), as described previously [30]. Northern Blot analyses

were performed using full-length Tnrip-1 coding cDNA as the probe, as described previously [13]. The same blots were then reprobed with glyceraldedyde-3-phosphate dehydrogenase (*GAPDH*) as a loading control. *In situ* hybridization was performed as described previously [13]. Sense or antisense ^{35}S-labeled cRNA probes were generated using appropriate polymerases from a mouse-specific cDNA for *Tnrip-1* (154-657 nucleotides of 0610009K11Rik). Quantitative RT-PCR (QRT-PCR) analyses were performed as described previously [30]. The QRT-PCR primers and Taqman® probes for *Tnrip-1* are primers 5′-GAAGGAGCTGTGCGGTCTGT-3′ and 5′-GGATCACCCCCTTGCAGTT-3′, and probe 5′-FAM- AAAGAAACACTCAACAGCCAGTTCGTGGA -TAMRA-3′ (Biosource International, Inc. Camarillo, CA), respectively.

Anti-Tnrip-1 polyclonal antibodies

Recombinant His-tagged truncated Tnrip-1 protein (amino acid 40—187) was generated in *E. coli*, purified with Qiagen nickel-chelating resin (QIAGEN Inc., Valencia, CA), and then injected into rabbits to generate anti-Tnrip-1 polyclonal antibodies. Pre-immune and hyper-immune sera were collected and passed through HiTrap Protein A HP affinity columns (Amersham Biosciences, Piscataway, NJ) to generate rabbit pre-immune IgG and anti-Tnrip-1 IgG, respectively.

Western blot analyses

Western blot analyses were performed as described previously [16;29]. Membrane blots were then reprobed with anti-actin antibodies (Sigma). Experiments were repeated three times on samples from at least three animals.

Immunohistochemical studies

Immunohistochemical studies were performed using the Rabbit ImmunoCruz Staining System (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's protocol. Experiments were repeated three times on samples from at least three animals.

Co-immunoprecipitation (Co-IP) assays

Homogenates of adult testes (2.1 mg proteins) were incubated with 1.2 μg of purified anti-GCNF, anti-Tnrip-1 antibodies, or their respective pre-immune serum IgG [16]. GCNF- or Tnrip-1 interacting proteins were precipitated by Protein A Sepharose (GE Healthcare Biosciences Corp., Piscataway, NJ) according to the manufacturer protocol, and then subjected to Western blot analyses using anti-Tnrip-1 or GCNF antibodies. Experiments were repeated twice on independent testicular homogenates.

Results

Restricted expression of Tnrip-1 in mouse testes

Using an *in silico* analyses, we identified a CoRNR-containing protein, 0610009K11Rik (http://www.ensembl.org/Mus_musculus/geneview?gene=ENSMUSG00000041241). We have named this gene testis-specific nuclear receptor-interacting protein-1 *(Tnrip-1)*. Computer analysis of *Tnrip-1* expression in mouse tissues showed that *Tnrip-1* is predominantly expressed in mouse testis (http://symatlas.gnf.org/SymAtlas) [26]. By RT-PCR, a specific Tnrip-1 DNA band was detected only in testes but not other tested tissues (Figure 1A). Northern blot analyses showed that 1.3 kb *Tnrip-1* transcript is restrictedly expressed in mouse testes (lane T, Figure 1B), but not in other tested tissues (Figure 1B).

Expression of Tnrip-1 in mouse testes during postnatal development

QRT-PCR analyses were performed to determine *Tnrip-1* expression levels in mouse testes at day 5, 9, 12, 14, 21, 28 and 56 after birth. As shown in Figure 2A, low levels of Tnrip-1 mRNA were detected in the testes of 5-12-day-old mice. In contrast, high levels of Tnrip-1 mRNA were detected in the testes of 14-56-day-old mice (Figure 2A).

Tnrip-1 mRNA in pachytene spermatocytes and spermatids

To determine *Tnrip-1* expression within the adult mouse testis, we performed *in situ* hybridization studies on testicular sections using S^{35}-radiolabelled Tnrip-1 sense or antisense probes. As expected, no specific radioactive signals in the testes were detected by the sense Tnrip-1 probe (Figure 2B). However, positive radioactive signals were detected in pachytene spermatocytes and round spermatids within seminiferous tubules by the antisense Tnrip-1 probe (Figure 2B). Few or close to none signals were observed in interstitial cells or spermatogonia within the testis (Figure 2B).

Tnrip-1 protein expression in adult mouse testes

To determine whether Tnrip-1 is expressed in adult mouse testes at the protein level, we generated anti-Tnrip-1 antibodies and performed Western blot analyses of homogenates from various tissues of adult mice. As shown in Figure 3A, a 42-kDa Tnrip-1 protein band was detected in adult testes, but not in other tissues. To determine the localization of Tnrip-1 protein in adult mouse testis, we performed immunohistochemical studies and found that positive signals were detected in the nuclei of pachytene spermatocytes and round spermatids by anti-Tnrip-1 antibodies, but not by preimmune normal rabbit IgG (Figure 3B).

Interaction of Tnrip-1 with GCNF in adult testes

Co-IP experiments were performed to determine whether Tnrip-1 can interact with GCNF in adult mouse testes. As shown in Figure 4, 42 kDa Tnrip-1 proteins in the homogenates of testes were co-precipitated by anti-GCNF antibodies, but not their preimmune serum IgG. Similarly, GCNF protein was pulled down by anti-Tnrip-1 antibodies, but not their preimmune serum IgG. However, similar amounts of GCNF and Tnrip-1 proteins in the samples used for Co-IP experiments were detected by anti-GCNF and anti-Tnrip-1 antibodies, respectively (Figure 4).

Discussion

Identification of nuclear receptor-interacting proteins in a specific tissue is important to understand their signaling pathways. GCNF is an orphan nuclear receptor that is specifically expressed in spermatogenic cells within the testis [13;14;16]. Previous studies have shown that GCNF functions as a transcription repressor to regulate transcription of protamine genes in a spermatogenic cell line [21]. It has been reported that GCNF can interact with other proteins to form a large protein complex and represses gene transcription in non-spermatogenic P19 cells [6;12]. It is possible that a similar scenario will occur in spermatogenic cells in which GCNF may recruit cofactors (coactivators or corepressors) to regulate its downstream target gene expression. It has been well documented that nuclear receptor corepressors contain I/LxxI/VI motifs, also called the CoRNR box [27;28]. During this post-genome era, putative testis-specific nuclear receptor corepressors can be identified by an *in silico* search of the CoRNR box from cDNAs enriched in mouse testis in mouse genome databases. Using this *in silico* approach, we identified a CoRNR-containing protein Tnrip-1. Consistent with our prediction, Tnrip-1 was indeed a testis-specific factor (Figure 1). Low levels of *Tnrip-1* mRNA in testes from 12-day-old or younger mice along with high expression levels of *Tnrip-1* mRNA in testes of 14-day-old or older mice (Figure 2A) indicates that *Tnrip-1* expression in the testis is developmentally regulated. Sine pachytene spermatocytes are present in 14-day-old or older

testes but not 12-day-old testes [31], *Tnrip-1* is likely highly expressed in pachytene spermatocytes. Indeed, robust *Tnrip-1* mRNA signals were detected in pachytene spermatocytes as well as spermatids within adult mouse testes by *in situ* hybridization (Figure 2B). In agreement with its mRNA expression, Tnrip-1 protein was detected only in the homogenates of adult mouse testes (Figure 3A). In addition, we found that Tnrip-1 is a nuclear protein and predominantly locates in pachytene spermatocytes and round spermatids (Figure 3B). Previous studies have shown that GCNF protein is expressed in mouse spermatocytes [32] and round spermatids [16;20;32]. Expression of Tnrip-1 protein in pachytene spermatocytes and spermatids (Figure 3B) correlates well with GCNF protein expression [16;20;32]. Since Tnrip-1 was identified as a putative nuclear receptor-interacting protein by our described *in silico* approach, we predicted that Tnrip-1 will interact with GCNF in the testis. In fact, we found endogenous Tnrip-1 protein can interact with GCNF in the testis, as demonstrated by Co-IP analyses (Figure 4). Collectively, we have successfully identified a novel testis-specific and GCNF-interacting protein Tnrip-1 in adult mice.

Since GCNF is involved in regulating the promoter activities of mouse protamine genes in spermatogenic GC-1 cells [21] and protamines are required for normal spermatogenesis and male fertility [18;19], Tnrip-1 will likely play a role in the modulation of GCNF-mediated protamine gene transcription in spermatogenic cells, affecting male fertility. It is interesting to note that Tnrip-1 contains a ring finger domain [33;34]. Ring finger domain-containing proteins (ring finger proteins) are involved in a number of biological processes such as transcriptional regulation of gene expression, modification of chromatin, cell proliferation and differentiation, tumorigenesis, and DNA damage repair [33;35]. Recent mouse genetic studies have shown that several ring finger proteins, such as Siah1a and Neural, play important roles during spermatogenesis [36;37]. In addition, many ring finger proteins (*e.g.* SNURF, ARA54 and Siah1a) can interact with nuclear receptors and some of them can function as nuclear receptor coregulators [38;39;40]. In the testis, several other nuclear receptors such as liver receptor homolog-1 (LRH-1) and receptors for retinoid acids (RARα, RARβ, RXRα and RXRγ) are also expressed in spermatogenic cells [41;42;43]. RARα and RXRβ are required for normal spermatogenesis [44;45;46], whereas LRH-1 appears to be involved in gene regulation in spermatogenic cells [42]. It is possible that Tnrip-1 will interact with these nuclear receptors in spermatogenic cells to regulate their downstream target gene expression, affecting male fertility. Further molecular analyses of Tnrip-1 in modulation of GCNF-mediated protamine gene promoter activities and other nuclear receptor-mediated gene transcription in spermatogenic cells as well as mouse embryonic stem cell knockout studies of Tnrip-1 will uncover its functional role in the testis.

In conclusion, we have identified a putative nuclear receptor-interacting protein Tnrip-1 in adult testes using an *in silico* approach. Moreover, our experimental studies showed that *Tnrip-1* is indeed a testis-specific gene and can interact with an orphan nuclear receptor GCNF in the testis. This study may serve as an example of identification of novel tissue-specific cofactors of proteins of interest (*e.g.* nuclear receptors) in an easy and fast manner during this post-genome era.

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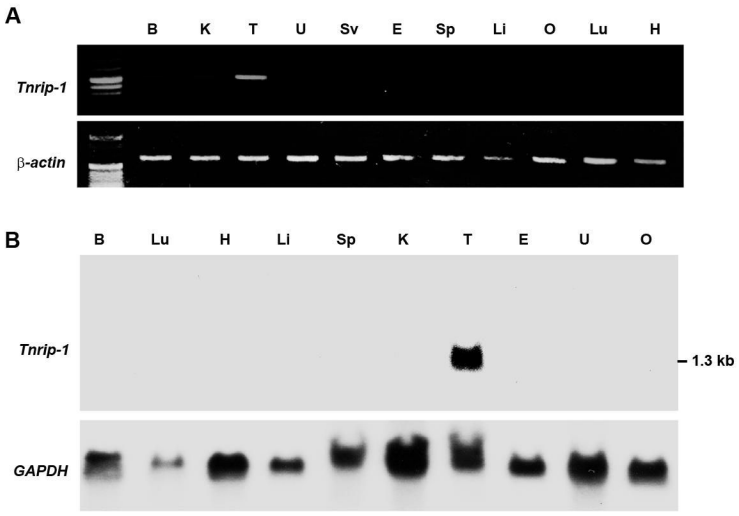

Figure 1.

Restricted expression of *Tnrip-1* in adult mouse testes by **(A)** RT-PCR and **(C)** Northern blot analyses. Lanes: B, brain; K, kidney; T, testis; U, uterus; Sv, seminal vesicles; E, epididymis; Sp, spleen; Li, liver; O, ovary; Lu, lung; H, heart.

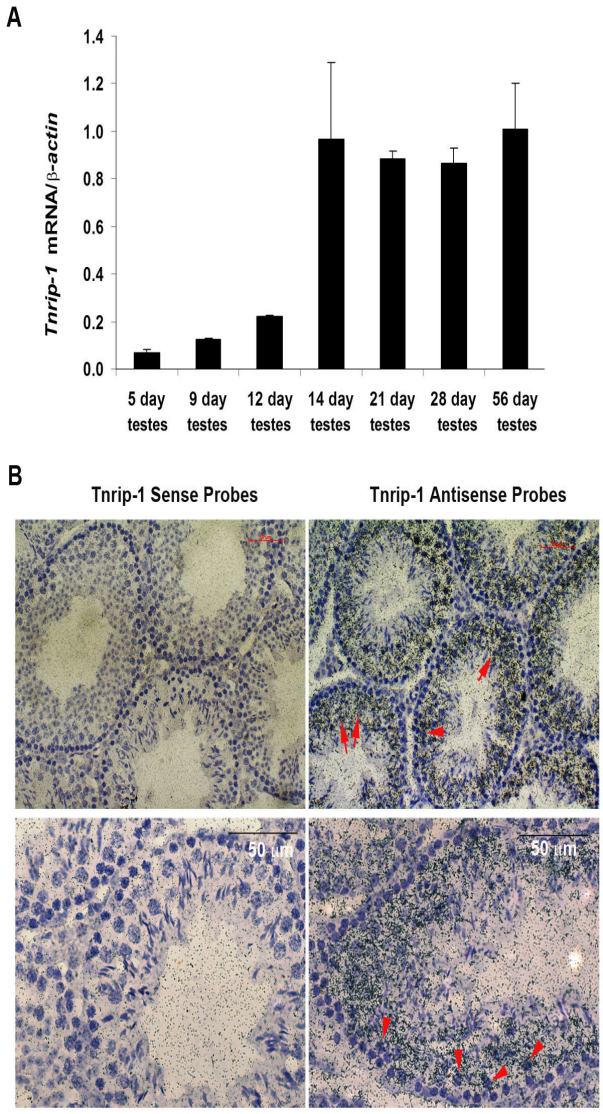

Figure 2.

Tnrip-1 expression in mouse testes during postnatal development and its predominant expression in spermatogenic cells of adult mouse testes. **(A)** QRT-PCR analyses showing the developmentally regulated *Tnrip-1* expression in mouse testes. Tnrip-1 mRNA levels from mice at various days after birth were normalized by *β-actin* mRNA in each samples (mean ± standard errors, n=3). **(B)** *In situ* hybridization studies showing the predominant *Tnrip-1* expression in pachytene spermatocytes (arrowheads) and round spermatids (arrows) within adult mouse testes.

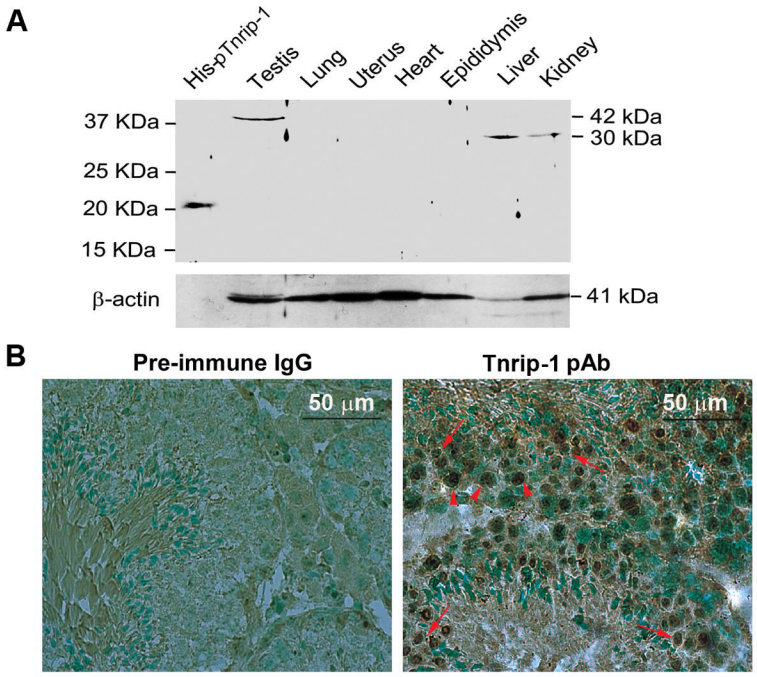

Figure 3.

Tnrip-1 protein expression in adult mouse testes. **(A)** Western blot analyses showing the restricted expression of Tnrip-1 protein (42 kDa) in the homogenates of adult mouse testes. **(B)** Immnohistochemical studies showing the expression of Tnrip-1 protein in pachytene spermatocytes (arrowheads) and round spermatids (arrows) in adult mouse testes.

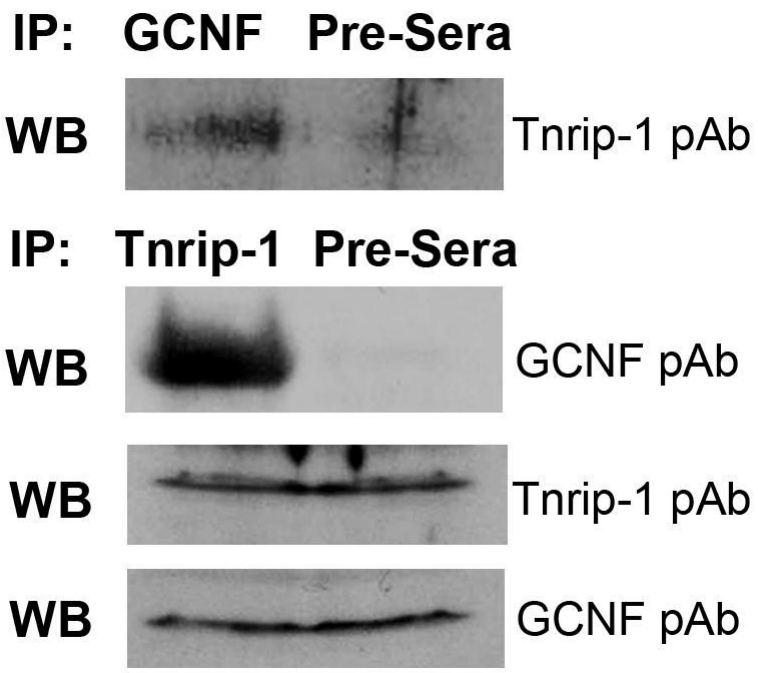

Figure 4.

Protein-protein interaction between Tnrip-1 and GCNF in the homogenates of adult mouse testes by Co-IP experiments. Top two panels: equal amounts of testicular proteins were immunoprecipitated (IP) with anti-GCNF antibodies, anti-Tnrip-1 antibodies or their respective preimmune serum IgG (Pre-Sera IgG). Precipitates were then subjected to Western blot analyses (WB) using anti-Tnrip-1 antibodies or GCNF antibodies. Bottom two panels: testicular homogenates for the above Co-IP experiments were probed with anti-Tnrip-1 or anti-GCNF antibodies to show the presence of both Tnrip-1 and GCNF proteins in the samples.