Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is a transport protein involved in gene specific mRNA export and protein translation during spermatogenesis

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Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25), a member of the DEAD-box protein family, is essential for completion of spermatogenesis. GRTH is present in the cytoplasm and nucleus of meiotic spermatocytes and round spermatids, and functions as a component of mRNP particles, implicating its post-transcriptional regulatory roles in germ cells. In this study, GRTH antibodies specific to N- or C-terminal sequences showed differential subcellular expression of GRTH 56 and 61 kDa species in nucleus and cytoplasm, respectively, of rodent testis and transfected COS1 cells. The 56 kDa nuclear species interacted with CRM1 and participated in mRNA transport. The phosphorylated cytoplasmic 61 kDa species was associated with polyribosomes. Confocal studies on COS-1 cells showed that GRTH-GFP was retained in the nucleus by treatment with a RNA polymerase inhibitor or the nuclear protein export inhibitor. This indicated that GRTH is a shuttling protein associated with RNA export. The N-terminal Leucine rich region (61 to 74 aa) was identified as the nuclear export signal that participated in CRM1-dependent nuclear export pathway. Deletion analysis identified a 14 aa GRTH sequence (100 to 114 aa) as a nuclear localization signal. GRTH selectively regulated the translation of specific genes including Histone 4 and HMG2 in germ cells. In addition, GRTH participated in the nuclear export of RNA messages (PGK2, tACE and Tp2) in a gene-specific manner. These studies strongly indicate that the mammalian GRTH/Ddx25 gene is a multifunctional RNA helicase that is an essential regulator of sperm maturation.

Modulation of RNA structure by members of the DEAD-box family of RNA helicases is a crucial step in many fundamental biological processes (1-7). This class of proteins participates in several aspects of RNA metabolism and translational events, including pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation and RNA decay, that ultimately regulate organelle gene expression for specific biological functions. RNA helicases contain at least eight conserved motifs, some of which have recognized functions such as ATP hydrolysis, RNA binding, and RNA unwinding that modulate RNA activity, while their N- or C-terminal extensions may confer substrate specificity.

Gene expression in germ cells requires temporal uncoupling of transcription and translation (8,9). Two-thirds of the mRNAs in the adult mammalian testis are associated with specific proteins to form messenger ribonuclear protein (mRNP) particles, and are stored in the cytoplasm of spermatids for translation at specific times required for progression and completion of spermatogenesis. We have recently discovered and characterized a hormone-dependent testis-specific RNA helicase in Leydig and germinal cells (meiotic
spermatocytes and spermatids) of rat, mouse and human termed Gonadotropin-Regulated Testicular Helicase (GRTH/Ddx25) (10). This enzyme is present in the nucleus and cytoplasm of germ cells, and selectively binds mRNA species as an integral component of messenger RNP particles with storage in chromatoid bodies located in the cytoplasm of spermatids (11). GRTH-targeted null male mice were sterile due to spermatid arrest at step 8 of spermatogenesis, with marked diminution of chromatoid bodies and failure to elongate. Since transcription of messages in spermatids steps-1 to 8 of these mice was not altered, we proposed that the function of GRTH is post-transcriptional.

GRTH protein is cell-specific and is hormonally regulated in the testis (12). Three species of GRTH protein, resulting from alternative utilization of translation initiation codons, were observed in the rat testis. Germ cells (round spermatid and spermatocyte) primarily utilized the 1st ATG codon (+1) and contained major proteins of 61/56 kDa, whereas interstitial Leydig cells preferentially utilized the 2nd ATG codon (+343) with expression of 48/43 kDa species. In round spermatids, hCG caused a significant decrease of 61 kDa species and an induction of 48/43 kDa. This is prevented by the androgen receptor antagonist, Flutamide, suggesting a role of androgen in utilization of the second ATG. The germ cell-specific 61/56 kDa species is essential for spermatogenesis.

To further define the molecular trajectory of GRTH as a RNA binding protein, and its translational function during sperm development, we examined the shuttling properties and structural requirements for GRTH protein/mRNA transport between nucleus and cytoplasm of transfected cells and testicular germ cells. Our findings indicate that GRTH has multiple regulatory roles at the RNA export and translational levels during germ cell development.

**Experimental Procedures**

**Animals** - Adult male rats (SD, Charles River Laboratories Inc, Wilmington, MA) and GRTH wild type and GRTH mice were housed in temperature and light-controlled conditions. All animal studies were approved by the National Institute of Child Health and Human Development Animal and Care and Use Committee. Animals were killed by asphyxiation with CO₂ and decapitated. Testes were removed and decapsulated for protein and RNA extraction. Germinal cells from seminiferous tubules were prepared and further purified for protein extraction.

**Testicular Cells Preparation** - Testicular germ cells were prepared by collagenase/ trypsin dispersion and purified by centrifugal elutriation (13).

**DNA constructs** - Full length of GRTH-pBK-CMV and GRTH-EGFP fusion constructs were prepared as previously described (12). Fusion constructs of EGFP with N-terminal fragments of GRTH (residues 1-59 aa, 1-80 aa, 1-100 aa and 1-114 aa) were generated by PCR using GRTH cDNA as template. These were subsequently subcloned at EcoRI/Smal sites of pEGFP-C1 vector (Clontech, Palo Alto, CA). PCR-based site-directed mutagenesis using EGFP-GRTH (1-114 aa) as template was performed using QuickChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All the constructs were verified by DNA sequencing.

**Cell transfection and confocal analysis** - In 6-well plate, constructs (2 μg) were transfected into COS-1 cells (80 % confluent) by Lipofectamine (Invitrogen, Carlsbad, CA). Cells were harvested for either fractionation of individual cellular compartment or directly used for Western blot analyses (see below). In confocal microscopy studies, COS-1 cells were transfected with the full-length of GRTH-EGFP fusion construct for 24 h and further treated for 3 hr with 90 μM DRB (5,6-Dichloro-1-β-D- ribofuranosyl- benzimidazole, RNA polymerase inhibitor) (Calbiochem, La Jolla, CA) or 100 nM LMB (Leptomycin B) (Calbiochem), nuclear protein export inhibitor. Cells were fixed,
counter-stained by Hoechst 33342, and observed under Bio-Rad laser confocal microscope system (MRC-1024). **Cellular compartment fractionation** - Nuclei and cytoplasm extracts of COS-1 cells, germinal cells and testis tissue were prepared using nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL) in presence of a cocktail of protease inhibitors (Roche, Basel, Switzerland). Cytoplasmic fractionation was performed using sucrose gradient sedimentation. Briefly, rat or mouse testes were homogenized in hypotonic buffer A (25 mM Tris, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT) containing protease inhibitors and cycloheximide (100µg/ml) using a Dounce homogenizer. Lysates were centrifuged at 500 g for 5 min to separate nuclear from cytoplasmic fraction. Sucrose gradients (7-47%) in buffer A or buffer A modified were prepared using a gradient mixer (Biocomp, Fredericton, N.B. Canada). For the EDTA condition employed, MgCl₂ in buffer A was replaced by 30 mM EDTA (buffer A modified) and used for the preparation of the cytoplasmic fraction and sucrose gradients. The cytoplasmic fraction (20 OD₂₆₀) was applied onto the linear sucrose gradient (7-47%) and subsequently centrifuged at 260,000 g (Beckman SW41 rotor) for 150 min. Fifteen 800 μl fractions were collected using an Isco density gradient fractionator (Teledyne Isco, Inc, Lincoln NE) equipped with a 5 mm pathlength density gradient flow cell, and a UA-6 UV/Vis detector with built-in chart recorder. Protein was precipitated by addition of 1 ml dry-ice cold ethyl alcohol to each fraction and these were kept frozen overnight at -20°C. Pellets obtained after centrifugation in a microcentrifuge (Eppendorf 5417R) at 20817g for 30 min at 4°C, were washed with 1ml ethyl alcohol and centrifuged for 5 min at 4°C. Pellets dried in a Speed vac concentrator were resuspended in 40 μl of 2X SDS protein gel solution and used for Western analysis. **Analysis of phospho-modification** - Testicular homogenates were applied to a phosphoprotein affinity column (BD biosciences, Palo Alto, CA) for enrichment of the phosphoprotein pool. The presence of GRTH in the phosphoprotein extract (5 μg) was revealed by Western blot analysis utilizing a GRTH specific antibody (see next section) and compared to original testicular homogenates. Calf intestinal alkaline phosphatase (CIP, 20 and 40 U, Promega, Madison, Wi) was added to testicular homogenates (120 μg/100μl) in the buffer containing 10 mM NaCl, 5 mM Tris-HCl, 1 mM MgCl₂, 1 mM DTT, protease inhibitor and incubated for 8 hrs at 37°C. 30 μg protein (1/4 of total reaction volume) was subsequently evaluated by Western analysis. COS-1 cells transfected with full-length GRTH cDNA were treated with protein kinase inhibitors (Calbiochem) including H-7:1-(5 - isoquinolinesulfonyl)- 2- methyl-piperazine (5, 15 μM); H-8: N-[2- (methylamino) ethyl]- 5-isoquinoline sulfonamide (5, 20 μM): H-89: N-[2- (p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide, 2HCL (10 μM) for 24 hrs, and cell lysates were subjected to Western Blot analysis. cAMP (8 bromo-cAMP) (Sigma Aldrich, St. Louis, Mo) were added to COS-1 cells transfected with GRTH and incubated for 24 hours. To evaluate the specificity of the effect of protein kinase A (PKA) on GRTH phosphorylation, COS-1 cells were cotransfected with plasmids expressing GRTH alone or with PKA catalytic subunit α (14) and/or rabbit muscle protein kinase inhibitor (PKI) (15) (2 μg each) (kindly provided by Dr. R. Maurer, Oregon Health Sciences University, Portland, Oregon). Cell extracts were assessed by Western blots utilizing GRTH antibody. To determine the phosphorylation status of the GRTH protein in whole testis and specific cellular compartments, total testicular lysate, purified nuclei and cytoplasm from rat and mouse testis were first immunoprecipitated by GRTH antibody and subsequently subjected to Western blot analysis for evaluation of phospho-amino acids with specific phospho-antibodies (see below).
Western Blot analysis - Protein extracts separated by 4-20% SDS-PAGE gel, and transferred to nitrocellulose membrane were assessed using the specific GRTH antibodies indicated in each study. Two rabbit anti-GRTH polyclonal antibodies were raised against either N-terminal (aa. 12-23) or C-terminal (aa. 466-479) peptides. Both antibodies were purified by peptide affinity chromatography utilizing GRTH peptides coupled to CN-Br-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ). Anti-phosphoserine (Sigma), and anti-phosphotyrosine and -threonine monoclonal antibodies (Cell Signalling Technology Inc, Denver, MA) were used to evaluate the phosphorylation of the GRTH protein. Anti-ribosomal protein S6, anti-Smac, and Anti-HDAC1 antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, CA) were used for verification of purified polyribosomal, cytoplasmic and nuclear preparations, respectively. Anti-Histone 4 (H4, Cell Signaling Technology Inc.), anti-phosphoglycerate kinase 2 (PGK1/2), high mobility group protein-2 (HMG2), testicular specific angiotensin converting enzyme (tACE), p68 (Santa Cruz Biotechnology Inc), Proacrosin (Novus Biologicals, Littleton, CO), β-tubulin (Santa Cruz) and β-actin (Sigma) were used in the designated experiments. Immunosignals were detected by a super-signal chemiluminescence system (Pierce Biotechnology, Rockford, IL). Specific PL10 rabbit peptide polyclonal antibody raised to aminoacids 28-38 of mouse PL10 (16) was purified by Protein A Sepharose (Amersham Biosciences, Piscataway, NJ). Antibodies were used at dilution 1:500 to 1:1000 and the appropriate 2nd antibody was employed (goat anti-rabbit and anti-mouse IgG HRP at 1:10,000; donkey anti-goat IgG HRP 1:5000). Immunosignals were detected by super-signal chemiluminescent system (Pierce).

Northern analysis - Total RNA from either whole cell or cytoplasmic fraction of adult mouse testis (GRTH+/+ and GRTH−/−) (11) was isolated using Trizol LS reagent (Invitrogen, Carlsbad, CA). 5 μg RNA samples were resolved on 1% agarose gel and transferred to nylon membranes. Mouse full length cDNAs including histone 4 (H4, Acc# NM_175652), high mobility group protein 2 (HMG2, Ac. # X67668), phosphoglycerate kinase 2 (PGK2, NM_031190), acrosin (Acr, NM_013455), testicular angiotensin converting enzyme (tACE, NM_009598), transition protein 2 (TP2, NM_013694) and PL10 (J04847) were subcloned by reverse transcription (RT) using oligo(dT) and PCR by gene specific primer pairs and sequenced. Full-length cDNAs of individual genes were used as probes for hybridization, except for tACE, where a specific exon-1 sequence was used. Signals were quantified by PhosphorImager analysis and normalized by using β-actin as the probe. Results are expressed as mean ± SE of at least three experiments.

Immunoprecipitation analysis - Immunoprecipitation (IP) of the GRTH-associated protein complexes was performed using the ProFound Co-Immunoprecipitation Kit (Pierce) by either affinity-purified anti-GRTH antiserum (C-terminus) or CRM1 (Santa Cruz). In this study, 100 μg primary antibodies or rabbit IgG were first immobilized to the antibody coupling gel followed by incubation with either 300 μg of rat testicular, nuclear or cytoplasmic extracts. The precipitated protein complex was recovered by brief centrifugation, followed by three sequential washes. The complex was eluted for Western blot analysis.

Results

Subcellular localization and phospho-modification of GRTH in rodent testis. The subcellular distribution of endogenous GRTH protein species from mouse testis and overexpressed full- length GRTH cDNA in COS-1 cells was assessed by Western analysis using C- and N-terminal GRTH peptide antibodies (Fig. 1 A, left- above and below panels). Both antibodies revealed two major GRTH protein species (61 and 56 kDa) that were differentially distributed in the cellular compartments of mouse testis.
The 61 kDa protein species was primarily present in the cytoplasm while the 56 kDa species was found predominantly in the nucleus. Both of these protein species were absent in GRTH null mice. Over-expressed GRTH protein in COS-1 cells revealed a similar pattern of subcellular distribution as the endogenous testicular GRTH protein. (Fig. 1A, right) and similar results were observed in the rat (not shown). Since both GRTH antibodies showed comparable results, the antibody to the C-terminal peptide was used in the subsequent studies. Only the testicular 61 kDa but not 56 kDa protein species or β-actin was enriched by phosphoprotein affinity column fractionation, suggesting that the 61 kDa protein was a phosphoprotein (Fig. 1B-left panel). The phospho-form nature of this 61 kDa species was also indicated by its conversion to the lower molecular weight species (56 kDa) after treatment with calf intestinal alkaline phosphatase (CIP) (Fig. 1B-middle panel). This was supported by the finding of a reduction of the 61 kDa species to 56 kDa in COS1 cells transfected with full length GRTH cDNA after the treatment with protein kinase inhibitors including H7, H8 and H89 (Fig. 1B-right panel). In contrast, the 61 kDa species was enhanced by cAMP treatment of cells expressing GRTH (Fig. 1 D-left panel). This protein species was significantly increased by over-expressing the catalytic subunit of protein kinase A (PKAα), and the stimulation was prevented by coexpression of the PKA inhibitor (PKI) cDNA. In addition PKI inhibited the basal levels of the 61 kDa species which presumable is generated by basal endogenous levels of cAMP present in the cells (Fig. 1D-right panel).

The endogenous phosphorylation status of the GRTH protein in the rat and mouse testis was further determined by immunoprecipitation analysis (IP). Two protein species (56 and 61 kDa) were clearly observed in the rat total testicular lysate (T) prior to IP analysis (Fig. 1 C, right panel, lane 1). Following IP of the testis lysates by the GRTH antibody only the 61 kDa species was detected in Western blots by the phosphothreonine antibody (p-Thr) (Fig. 1 C, right panel, lane 3). No signal was observed when IgG was used as negative control (Fig. 1C, right panel, lane 2) or when antibodies to phospho-tyrosine or -serine were used (not shown). Similar results were found in mice total testicular lysates where the GRTH protein was phosphorylated at the threonine not at the Tyr or Ser residues (Fig. 1 C, left panel, lanes 2 and 4 vs lanes 6 and 8). In the mouse, in contrast to the rat because of its low abundance the 56 kDa species (non-phosphorylated) was difficult to detect in testis homogenates but was observed in nuclei fractions. Consistent with our early results (Fig. 1 A and B), the 61 kDa Thr phosphorylated GRTH species was present in the cytoplasm, not in the nuclear compartment (Fig. 1C, lane 4 vs 6 in mouse, middle panel) and rat (not shown).

**Nuclear localization of overexpressed GRTH-GFP in COS-1 cells after treatment with Leptomycin B (LMB) and RNA polymerase inhibitor (DRB).** To gain insights into the functional association of GRTH protein association with individual cellular compartments, we initially investigated by confocal microscopy whether GRTH participates in a shuttling mechanism between nuclear and cytoplasmic compartments in COS-1 cells overexpressing GRTH-GFP fusion protein. As we previously reported in control cells, the GRTH-GFP fusion protein was present in both cytoplasm and nucleus (Fig. 2, left panel, top) (11). In this study, we showed that GRTH-GFP in nuclei after the treatment of transfected cells with nuclear protein export inhibitor (LMB) (Fig. 2, middle panel) and RNA polymerase inhibitor (DRB) (Fig. 2, lower panel).

**Leucine rich sequence (aa 61-74) of GRTH/Ddx25 functions as the nuclear export signal (NES) and participates CRM1-dependent nuclear export pathway.** Since GRTH was found to act as a transport protein between the nucleus and cytoplasm (Fig. 2), we examined whether GRTH interact with chromosome region maintenance-1 protein (CRM1), which is
known to be involved in the nuclear export pathway (17). CRM1 was abundantly expressed in the rodent testis as revealed by Western analysis (Fig. 3A, top, left). Immunoprecipitation (IP) of mouse testis extracts using GRTH antibody demonstrated its association with CRM1 as shown by Western blot (Fig. 3A, top panel, right). Similarly the GRTH protein was present in the complex when the IP procedure was performed using CRM1 antibody followed by Western analysis with GRTH antibody of both rodent species (Fig. 3A, middle panel, lanes 1 and 2 vs. 3) and CRM1 was shown to be associated with the 56 kDa nuclear species (non-phosphorylated GRTH) when compared to GRTH protein species present in the rat testicular homogenate (Fig. 3A, middle panel, lane 4) or purified fraction of nuclear (N) and cytoplasmic (C) compartment (Fig. 3A, middle panel, lanes 5 and 6). Furthermore, the CRM1-immunoprecipitation analysis (IP) of purified cellular compartment revealed that 56 kDa nuclear protein of both rodent species was associated with CRM1 (Fig. 3, lower right panel-rat, lane 3, left panel-mouse, lane 3) but not the cytosolic fraction (Fig. 3, lower right panel-rat; lane 2; left panel-mouse, lane 2). These results suggested that GRTH utilizes the CRM1-dependent nuclear export pathway for mRNA transport.

To identify the GRTH nuclear export signal (NES), we performed mutagenesis analysis on its N-terminal sequences. The 1-114 aa N-terminal sequence contains two leucine-rich stretches, one noted between 61-74 aa with mismatches when compared to the classical leucine-rich NES consensus (0X2-30X2-30X0, 0: hydrophobic residue; X: polar aa), and the other was located between 100-114 aa (Fig. 3B, below). Mutation of leucine residues at 66, 67 and 70 aa enhanced the expression of 114 aa fusion protein in the nucleus (66/67/70Δ). In contrast, mutation of leucine residues within 100-114 aa at 104,108 and 109 aa, significantly decreased the nuclear expression of the mutant EGFP-GRTH fusion protein (104/108/109Δ) (Fig. 3B, see also Fig. 4).

Localization of a Nuclear Localization Signal (NLS) of GRTH. To define in more detail the NLS, deletion and mutation analyses were performed in the N-terminal sequence of GRTH (1-114 aa). The GRTH-GFP fusion construct 1-114 aa and deletion constructs 1-59, 1-80, 1-100 and 1-114 aa were overexpressed in COS-1 cells (Fig. 4A). The GRTH protein containing the first 114 aa (EGFP-GRTH1-114 aa) was expressed in both cytoplasm and nucleus of COS-1 cells. Fusion constructs with deletions (EGFP-GRTH1-59 aa, EGFP-GRTH1-80 aa and EGFP-GRTH1-100 aa) revealed expression of their respective protein only in the cytoplasm. Mutation analysis of the 114 aa construct in the region between 101-114 aa showed that single or double mutation of two basic residues (R103→A, K105→A) in EGFP-GRTH (1-114 aa) fusion construct abolished the respective protein expression in the nucleus (Fig. 4B).

Cytoplasmic GRTH protein is associated with polyribosome and participates translational regulation of gene expression. We also investigated the functional role of cytoplasmic GRTH as translational regulator, by examining its potential association with polyribosomes which are required for translation of RNA messages. Mouse testicular homogenates were fractioned in 7-47 % continuous sucrose gradient followed by Western analysis of fractions using anti-GRTH (Fig. 5, [2] and [4]) and anti-ribosomal S6 (Fig. 5, [3] and [5]) antibodies. Optical density profiles of the gradient fractions were assessed by OD at 254 nm (Fig. 5, [1]). The lighter fractions of the gradient contained free ribosome subunits (40S, 60S) and monoribosomes (80S) and the heavy fractions polyribosomes. EDTA caused the dissociation of polyribosomes into free ribosome subunits (Fig. 5, [1]). Although both GRTH protein species (61/56 kDa) were localized in the lighter fraction of the gradient only the 61 kDa species was associated with polyribosomes (Fig. 5, [2]).
This was further indicated by the significant shift of GRTH protein cosedimenting with polyribosomes towards the lighter part of the gradient after EDTA treatment which causes dissociation of ribosomes from mRNA (Fig. 5, [1, 4, 5]). A similar pattern was observed in rat testicular homogenates (not shown).

**GRTH/Ddx 25 selectively modulates subsets of gene translation and RNA export from nucleus in germ cells during development.** The functional involvement of GRTH in translation was further implicated by the selective loss of protein expression in germ cells of the GRTH knockout mice. Genes expressed in the early stage of spermatogenesis, such as H4 and HMG2 (Fig. 6A, left), were not present in the purified spermatocytes of null mice. (Fig. 6 B, left). At later stages of germ cell development (Fig. 6A right), PGK2 and angiotensin-converting enzyme (tACE) and transition protein 2 (TP2) were absent in purified round spermatids of null mice (Fig. 6 B, right). However there was no change in the protein expression of the other genes examined, including P68, PL10 from spermatocytes, and acrosin (Acr) from round spermatids (Fig. 6A versus 6B), suggesting gene-specific translational regulation by GRTH during germ cell development. Taken together, with the evidence of GRTH association with polyribosomes these findings suggested an essential role of GRTH in the translational regulation of a subset of genes expressed during germ cell development.

The transcription of all genes expressed was not changed in the GRTH null mice as indicated from the unchanged steady-state total mRNA level (11). However, GRTH appears to participate in the nuclear export of specific genes (Fig. 6C, right). A similar cytoplasmic to total RNA ratios for H4 and HMG2 was observed in spermatocytes of wild type and GRTH−/− mice, indicating that their export was not altered and changes in protein expression could result from translational or processing events in the cytoplasm. In contrast, ratios of PGK2, tACE and Tp2 RNA species from round spermatid (RS) were significant reduced while those of acrosin (Acr) and PL10 were unchanged. Taken together, these results indicate that GRTH is an RNA export protein for PGK2, tACE and TP2 and participates in the translation for H4 and HMG2.

**Discussion**

The present study has demonstrated differential localization of two GRTH protein species in subcellular compartments of germ cells. These include the 56 kDa species, and the phosphorylated 61 kDa form primarily localized in the nucleus and cytoplasm respectively, of germ cells. Our studies indicated that cAMP-PKA participates in the post-translational modification of GRTH protein, increasing formation of the 61 kDa from the 56 kDa form. We have also provided evidence for dual functional roles of GRTH in RNA export from nucleus to cytoplasm and in the translation of specific RNA transcripts at specific stages in germ cell development.

Our previous studies suggested that GRTH functions as a transport protein that regulates shuttling of mRNP particles between the nucleus and the cytoplasm (11). Transport of macromolecules through the nuclear pore complexes has been shown in many cases to be regulated by phosphorylation events associated with the accessibility and affinity of cargo-signal recognition by importin/exportin (18). However, since GRTH is only expressed as a cytoplasmic phospho-form and the unphosphorylated species (56 kDa) was found to be associated with CRM1, it is likely that this post-translational modification is not related to mRNP export but rather to other cytoplasmic-related events (Fig. 5-6). It is possible that phosphorylation of GRTH could affect nuclear import (18) and/or have other functions such as inducing conformational changes to recruit protein(s) to gain access to chromatoid bodies, remodeling RNA-protein interactions and/or initiating translation of its target genes.
Shuttling of GRTH between the nucleus and cytoplasm was observed in living cells. The distribution of GRTH in nucleus and cytoplasm was altered when the synthesis and/or export of RNA was repressed. Accumulation of GRTH in the nucleus when mRNA synthesis was blocked, indicated that the GRTH shuttle is mRNA-dependent (Fig 2). Treatment of cells with the CRM1/ exportin 1 inhibitor, LMB, caused retention of GRTH in the nuclear compartment, suggesting that CRM1 is involved in GRTH nuclear export as a component of the mRNP particles. Immunoprecipitation analysis further revealed a physical interaction of CRM1 and GRTH (Fig. 3A). CRM1, an evolutionarily highly conserved protein, is known to be essential for nuclear RNP particles export through its binding to a leucine-rich sequence of target genes (17). There are two leucine-rich regions (aa 61-74 and aa 102-112) at the N-terminal of GRTH. Mutational analysis indicated the differential usage of these leucine sequences, with the first leucine-rich region, aa 61-74, functioning as a nuclear export sequence (NES) that could serve as the binding site to CRM1. However, the second leucine-rich region, aa 102-112, appears to function as a nuclear localization signal (NLS) (Figs. 3 and 4).

The majority of substrates, exported via CRM1, including DEAD-box RNA helicases, contain a short, leucine-rich NES. Most NESs bind to CRM1 with relatively low affinity, but this interaction is greatly stimulated by RanGTP (17). DEAD-box RNA helicase An3 bound CRM1-RanGTP through its N-terminal leucine-rich NES (19). Mutation of the leucine-rich NES of Xp54/Ddx6 or treatment of LMB inhibited nuclear export of this protein (20). Vasa/MVH, a germ cell-specific RNA helicase, interacts with RanBPM which recruits RanGTP. The resulting complex may play a role in trapping and transferring of specific RNAs from the nucleus to the chromatoid body (21). However it was noted that the binding of DDX3 to CRM1 did not required its N-terminal NES (rather a C-terminal sequences between aa. 260 and 517) and RanGTP (22) for HIV-1 Rev-PRE export. Transport of macromolecules through the nuclear pore is believed to depend on the association of RNA with a group of protein complexes. This has been well characterized in DBP5 (23,24), a GRTH closely related member of DEAD box family. Acting as an mRNA export factor, DBP5 is associated with Rat8p (25,26) and Nup159/Gle1/Gfd1/Nab2 complexes in the yeast (27,28). It is conceivable that GRTH is associated with any of the mammalian homologs mentioned above in the nuclear export process through the CRM1 participating pathway.

Spermatogenesis is a complex developmental progression that depends on a specific program of gene expression during mitotic, meiotic and haploid stages to produce mature germ cells. Studies using purified-stage specific cells (spermatocytes and round spermatids), together with the genetic targeted GRTH null mice model, have begun to clarify the regulatory mechanism of GRTH during germ cell development. Sucrose gradient fractionation experiments demonstrated that the cytoplasmic phosphorylated form of GRTH is tightly associated with polyribosomes, where the active translational process occurs (Fig. 5). From comparative protein profiles of enriched spermatocytes and round spermatids in wild type and GRTH knockout mice, we have identified some of the GRTH-targeted genes. These include histone 4 (H4) and high mobility group protein 2 (HMG2) in spermatocytes, and testicular specific glycolytic enzyme phosphoglycerate kinase 2 (PGK2), testicular isoform of angiotensin converting enzyme (tACE) and the transition protein 2 (TP2) in spermatids which are known to be important for germ cell development. Despite the disruption in the synthesis of these proteins, the expression of their corresponding RNA transcript was intact (11) in GRTH null mice testis.

Germ cell maturation is associated with particular protein expression at a specific stage of spermatogenesis
Expression of PGK2 and tACE and TP2 proteins was observed as early as in spermatocytes. PGK2, tACE were most abundant in round and elongating spermatids, while TP2 levels were highest in round spermatids (Fig. 6 A). The mRNA transcripts of these and other relevant genes must be stored as mRNP particles to await translation at specific times of development. The timing of GRTH protein expression in the pachytene spermatocyte, prior to the expression of PGK2, tACE and TP2 in spermatids (12), is consistent with the activation of translation in these genes triggered by GRTH. In addition to the translational regulatory function, GRTH may participate in the nuclear-cytoplasm RNA transport of specific set of mRNAs. It was noted that cytoplasmic RNA levels of PGK2, tACE and TP2, but not germ cell specific proacrosin (Acr) and other RNA helicases that are colocalized in pachytene spermatocytes including p68 and PL10, or house keeping gene β-actin, are reduced in the GRTH null testis (Fig. 6). This is consistent with earlier evidence discussed above that GRTH is required for the export of RNA-associated RNP particles (11). However, we cannot exclude the possibility that RNA degradation may cause the decrease in cytoplasmic RNA. The stability of a relevant RNA in the RNP complex could be changed in the absence of GRTH. On the other hand, the export of specific RNA could be partially compensated by the participation of other family members. This may occur during the progression from haploid spermatocytes to the spermatid arrest point in null mice (11). These results provide the first evidence of GRTH function in RNA export in a gene-specific manner. The greatly diminished size of chromatoid bodies in spermatids of GRTH null germ cells (11) is probably the result of decreased mRNA export/and or storage. On the other hand, H4 and HMG2 genes, which are effectively transcribed in GRTH null mice, displayed cytoplasmic versus total cellular mRNA ratios that were comparable to those of WT mice. However, the lack of expression of both proteins in null mice points to the essential role of GRTH in translation.

Diverse functionality of DEAD-box RNA helicases in RNA metabolism has been previously recognized. The eukaryotic initiation factor 4A (eIF4A), which was the first characterized RNA helicase, unwinds the secondary structures at the 5’ end of eukaryotic mRNAs for the scanning by the small ribosomal subunit (6). Xp54/Ddx6/ Dhh1, the first helicase linked to mRNA export, storage (20) and mRNA masking (32), is required for decapping and mRNA degradation in eukaryotes (33). Dbp5, an RNA exporting factor, remains associated with mRNA during protein synthesis (24). Vasa, a translational activator of germ cell mRNAs, mediates translation through interaction with the drosophila yIF2 homolog (34).

We now propose that GRTH/Ddx25 has multifunctional roles in the processing of germ cell-specific RNAs, and postulate a model of GRTH action in male germ cells during development. After its translation in germ cells, some GRTH protein is phosphorylated and may become associated with the translation machinery to regulate target gene expression. GRTH is also transported into the nucleus, where it selectively binds mRNAs and associates with other components of the mRNP complex. The RNA unwinding activity of GRTH could help to arrange RNAs into and/or within mRNPs. The unphosphorylated GRTH-bound mRNP particles are transported from the nucleus to the cytoplasm via CRM1 in the nuclear export pathway. As a component of mRNP particles, some of the GRTH-associated specific messages are directly engaged with the polyribosomal translational machinery. Others are initially stored in subcellular organelles such as the chromatoid body, and are subsequently released for translation at specific times during spermatogenesis. The function of GRTH in RNA export and protein translation highlights its basic molecular regulatory mechanism and its essential role during spermatogenesis.
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Footnotes

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The abbreviations used are: GRTH, GRTH/Ddx25, Gonadotropin Regulated Testicular Helicase; tACE, testicular angiotensin converting enzyme; PGK 1/2, phosphoglycerate kinase 2; TP2 transition protein 2. PL10, Ddx10, CRM1, chromosome region maintenance-1 protein; PKA, protein kinase A. GFP, green fluorescent protein.

Figure Legends

Fig. 1. Subcellular localization and phospho-modification of GRTH in mouse testis. A. Western analyses were performed to assess GRTH protein expression in the nuclear (N) and cytoplasmic (C) compartment of mouse testis and COS 1 cells transfected with full length of GRTH-pBK-CMV. Antibodies against C-terminal and N-terminal GRTH peptides were used in the study. B. Phospho-modification of GRTH protein species. Enriched mouse testicular phosphoprotein using phosphoaffinity column (Post-enrich.) was compared to the original testicular homogenates (Pre-enrich.) by Western blot analysis (B-left panel). CIP: Calf intestinal alkaline phosphatase treatment (B-middle panel). Treatment with Kinase inhibitors (B-right panel). H7: 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H-8: N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide; H-89: N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide, 2HCL (10 μM). C. Western blot analysis of endogenous GRTH protein of rodent species in total testicular lysates (T) and purified cytoplasmic (C) or nuclear (N) fraction. IP: immunoprecipitation. Ab: specific antibody used for Western blot analysis: GRTH; p-Ser, p-Thr and p-Tyr, phospho-antibodies. D. Western blots of effect of cAMP treatment on COS-1 cells overexpressing GRTH (left panel, above). Western blots of modification of overexpressed GRTH protein species by co-transfected PKA α catalytic subunit and PKA inhibitor (PKI) (right panel, above) in COS-1 cells. Quantitation of results from three independent experiments such as the representative experiment shown in D, below.

Fig. 2. Cellular redistribution of GRTH in COS-1 cells after treatment with nuclear protein export inhibitor (LMB) and RNA polymerase II inhibitor (DRB). Over-expressed GRTH-EGFP fusion protein in COS-1 cells was examined by confocal microscope under a 40x oil objective lens. Green fluorescence signals from GRTH-EGFP (left), nuclear staining by Hoechst 33342 (middle) and merged images (right). Bar = 10 μm

Fig. 3. Leucine rich sequence (aa 61-74) of GRTH functions as the putative nuclear export signal and participates CRM1 dependent nuclear export pathway. A. Western blot (WB) of CRM1 was expressed in rodent testis (top panel, left). Immuno-precipitation (IP) of testicular proteins using a GRTH peptide antibody (top panel, right) or CRM1 antibody (middle and lower panels) followed by Western blot analysis using CRM1 and GRTH antibodies, respectively. Ab: antibody. Goat anti-rabbit IgG (IgG) : negative control. Testis: total testicular lysate. N: nuclear fraction. C:cytoplasmic fraction. B. Western analysis of over-expressed GRTH/Ddx25 fusion protein (EGFP-GRTH 114 aa) in cytoplasmic (C) or nuclear (N) compartment after transient
transfection in COS-1 cells. Δ: mutation of aa residue at designated location. β-actin was used as the protein loading reference. Nuclear HDAC1 and mitochondrial Smac proteins were used as the purity index of cell fractionation for nucleus and cytoplasm, respectively.

Fig. 4. Characterization of a nuclear localization signal of GRTH. Western analysis of a series N terminal deleted EGFP-GRTH fusion protein (aa. 59 to 114) in the nuclear (N) and cytoplasmic (C) compartment of transient transfected COS-1 cells. Δ: aa mutation from arginine (103) and lysine (105) to alanine. β-actin was used as the protein loading reference. Nuclear HDAC1 and mitochondria Smac proteins were used as the purity index of cell fractionation for nuclear and cytoplasm respectively.

Fig. 5. Cytoplasmic GRTH is associated with polyribosomes. Fractionation of adult mouse testicular extracts on a 7-47 % continuous sucrose gradient in the absence or presence of EDTA. Optical density profiles (OD 254 nm) of sucrose gradient fractions are shown in the top panel (panel 1). Location of ribosomal subunits (40S and 60S), monoribosomes (80S) indicated by arrow, and polyribosomes (bar). Each fraction was analyzed by Western blot using antibodies to GRTH (panels 2 and 4) or S6 ribosomal protein (panels 3 and 5).

Fig. 6. GRTH selectively modulates subsets of germ cells gene translation during development. A. Western blots of gene of interest in the germ cells of normal adult mice during development. Enriched spermatocytes (SP), round spermatids (RS) and elongating spermatids (ES) were obtained after centrifugal elutriation. B. Western analysis of gene of interest of germ cell specific gene expression in the wild type (+/+) and GRTH null (-/-) mice during development. C. GRTH selectively modulates subsets of germ cells RNA exporting from nuclear to cytoplasm. Northern blot analysis (Left panel) of cytoplasmic (Cyt) and whole cells (Total). RNA isolated from purified spermatocytes (SP) or round spermatids (RS) of wild type (+/+) and GRTH knockout (-/-) mice. Stage specific germ cells cDNA was used as the hybridization probe. Signals were quantified by phosphorimager and normalized by β-actin. The ratios of cytoplasmic to whole cell RNA of each specific gene were used to represent the RNA export efficiency from nucleus to cytoplasm. H4: Histone 4. HMG2: high mobility group protein 2. PGK2: phosphoglycerate kinase 2. tACE: testicular specific angiotensin converting enzyme. Acr: proacrosine. TP2: transition protein 2. PL10 (Ddx10).
Figure 1
Figure 2
Figure 3
Figure 4

A. 

| Protein            | C  | N  | C  | N  | C  | N  | C  | N  |
|--------------------|----|----|----|----|----|----|----|----|
| kDa                | 50 | 36 | 50 | 36 | 50 | 36 | 50 | 36 |
| GRTH               |    |    |    |    |    |    |    |    |
| Actin              |    |    |    |    |    |    |    |    |
| HDAC1              |    |    |    |    |    |    |    |    |
| Smac               |    |    |    |    |    |    |    |    |
| EGFP-GRTH^{1-59aa} |    |    |    |    |    |    |    |    |
| EGFP-GRTH^{1-80aa} |    |    |    |    |    |    |    |    |
| EGFP-GRTH^{1-100aa}|    |    |    |    |    |    |    |    |
| EGFP-GRTH^{1-114aa}|    |    |    |    |    |    |    |    |

B. 

| Treatment          | C  | N  | C  | N  | C  | N  | C  | N  |
|--------------------|----|----|----|----|----|----|----|----|
| kDa                | 50 |    |    |    |    |    |    |    |
| GRTH               |    |    |    |    |    |    |    |    |
| Actin              |    |    |    |    |    |    |    |    |
| HDAC1              |    |    |    |    |    |    |    |    |
| Smac               |    |    |    |    |    |    |    |    |

Control, 103Δ, 105Δ, 103Δ105Δ
Figure 5
Figure 6
Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is a transport protein involved in gene specific mRNA export and protein translation during spermatogenesis

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