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Identification of Messenger RNA Substrates for Mouse T-STAR

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Abstract—Using the method of isolation of specific nucleic acids associated with proteins (SNAAP), we have identified 10 candidate target mRNA substrates bound by mT-STAR (mouse T-STAR protein) from testis extract. Among them, our study focused on *Fabp9*, a gene that is essential for male gametogenesis, and showed that mT-STAR could directly bind to *Fabp9* mRNAs. The binding sites are in a short sequence of the coding region and 3′ untranslated region of *Fabp9* mRNA. These suggest that mT-STAR can regulate the metabolism and expression of *Fabp9*. In conclusion, identification of mT-STAR-bound mRNA substrates might help to illustrate the potential spectrum of the process and provide valuable insight into the biological function of this RNA-binding protein in spermatogenesis.

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Key words: *Fabp9*, mRNA substrates, mT-STAR, SNAAP, spermatogenesis

Spermatogenesis involves a series of dramatic molecular and morphological alternations during the development and maturation of male germ cells. This process is similar in all animals, and it is related to cell growth, division, differentiation, and signal transmission in spermatogenic cells. Therefore, a highly coordinated expression of genes is crucial for normal germ cell development [1]. RNA-binding proteins play an important role in spermatogenesis, and they are required not only in the cytoplasm but also in the nucleus. So far, some RNA-binding proteins and their mRNA substrates have been identified [2]. Among them, RBMY, expressed especially in testis, is probably the most important nuclear RNA-binding protein correlated with male infertility. Developmental stage-specific expression of RBMY suggests that it is involved in early phases of spermatogenesis [3]. *RBMY* is a multicopy gene in the long arm of the Y chromosome, and microdeletions of the region encoding active *RBMY* are related to spermatogenic disruption during meiosis [4]. The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of nuclear RNA-binding proteins [5]. They are subject to negative regulation in murine testicular germ cells: hnRNP A1 is turned off prior to meiosis, and the other hnRNPs are turned off subsequently [6]. HnRNP G-T, which is very similar to hnRNP G and RBMY, is expressed prominently in germ cells and particularly in the nuclei of meiotic spermatocytes. HnRNP G-T might regulate RNA processing as a germ cell-specific splicing regulator, or it might replace the function of hnRNP G protein during meiotic prophase [7, 8].

T-STAR is a novel member of the signal transduction and activation of RNA (STAR) family. STAR proteins have multiple functions in pre-mRNA splicing, signaling, and cell cycle control [7, 9, 10]. These processes might be very finely regulated during germ cell development. T-STAR was first identified in a yeast-two-hybrid screen as a protein interacting with RBMY in spermatogenesis. RBMY and other hnRNP G family members are candidate downstream targets for regulation by T-STAR. Venables et al. proposed a very attractive possibility that members of the hnRNP G family constitute a class of tissue-specific splicing enhancer proteins that are regulated by or binding alongside T-STAR [11]. T-STAR interact-

Abbreviations: DIG, digoxigenin; hnRNP, heterogeneous nuclear ribonucleoprotein; mT-STAR, mouse T-STAR (testis signal transduction and activation of RNA) protein; RBB, RNA binding buffer; SNAAP, specific nucleic acids associated with proteins.

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ed with two groups of proteins: STAR proteins (itself and SAM68) and members of the hnRNP G family (hnRNP G, RBMY, and hnRNP G-T). Expressed primarily in the testis, T-STAR can bind to RNAs through its KH RNA-binding domain, and it is involved in both homotypic and heterotypic protein–protein interactions [7, 11]. Both RBMY and hnRNP G interact with ubiquitously expressed proteins involved in splice site selection (SR proteins and Tra-2β) and signal transduction (T-STAR and SAM68).

This shows that T-STAR can play an important role in coupling signal transduction and cell-mRNA splicing in germ cells [11-13]. Although previous studies have revealed that T-STAR has multiple biological functions in many aspects, its mRNA substrates are still unclear. To elucidate the role of T-STAR in cellular functions and its molecular mechanism during spermatogenesis, it is essential to identify target mRNA substrates whose activity is regulated through the binding of T-STAR. In our study, 10 mRNA substrates were identified by mT-STAR through SNAAP screening and further confirmed by RT-PCR analysis.

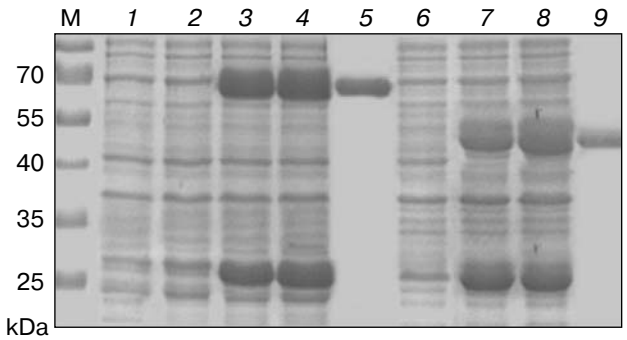

Fig. 1. SDS-PAGE analysis of the expression and purification products of GST-mT-STAR and GST-mNΔKH. Lanes: *1*) non-induced pGEX-5X-3; *2*) non-induced pGEX-5X-3-mT-STAR; *3*) induced pGEX-5X-3-mT-STAR; *4*) soluble protein extract containing GST-mT-STAR; *5*) purified GST-mT-STAR; *6*) non-induced pGEX-5X-3-mNΔKH; *7*) induced pGEX-5X-3-mNΔKH; *8*) soluble protein extracts containing GST-mNΔKH; *9*) purified GST-mNΔKH.

MATERIALS AND METHODS

Preparation of testis total extract. Testes were removed from 5-week-old Balb/C mice and placed into lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, protease inhibitor cocktail, 40 U/ml RNase OUT, and 0.5% Triton X-100) [14]. The tissue was diced with a razor blade and then sonicated. After centrifugation for 5 min at 4°C and 15,000*g*, supernatant containing proteins and RNAs was collected and frozen with 5% glycerol in aliquots at –70°C.

Preparation of glutathione S-transferase fusion proteins. The cDNAs for mT-STAR were prepared by PCR using a pair of primers (5′ primer, 5′-CGGGATCCC-CATGGAGGAGAAGTACCTGCC-3′, and 3′ primer, 5′-GCGTCGACAGGACGGTGGAGATTGGCTAT-3′) based on the reported sequence of the mouse protein. To construct the cDNAs of mNΔKH (mT-STAR without the RNA binding domain KH, amino acids from 58 to 177), the PCR products of A (5′ primer, 5′-CGGGATC-CCCATGGAGGAGAAGTACCTGCC-3′, and 3′ primer, 5′-CCGCTCGAGTCCCAGCTTCATGTTC-TTGTT-3′) and B (5′ primer, 5′-CCGCTCGAG TCA-GAAAATGCAGATGTCCCAG-3′, and 3′ primer, 5′-GCGTCGACAGGACGGTGGAGATTGGCTAT-3′) were both digested with *Xho*I, extracted, and ligated with T4 ligase. Then the cDNAs for mT-STAR and mNΔKH were digested with *Bam*HI and *Sal*I, and the resulting DNA fragments of 1091 and 735 bp were cloned into the *Bam*HI-*Sal*I sites of the PGEX-5X-3 vector to generate GST-mT-STAR and GST-mNΔKH expression plasmids. They were confirmed by analysis of restriction enzyme digestion and DNA sequencing. Then they were transformed in *E. coli* BL21 cells, respectively. The recombinant proteins were induced by addition of 1 mM isopropyl-β-D-thiogalactoside for 4 h at 24°C (Fig. 1). Cells were collected by centrifugation at 8000*g* for 15 min. After disruption by sonication in phosphate-buffered saline, insoluble matter was removed by centrifugation, and the supernatant containing the soluble proteins was finally collected.

SNAAP screen. The SNAAP screen (method for isolation of specific nucleic acids associated with proteins) was carried out as described by Trifillis et al. [15]. Micrococcal nuclease-treated GST, GST-mT-STAR, and GST-mNΔKH proteins were bound to 40 μl GST-beads at 4°C for 40 min in a total volume of 1 ml in RBB (RNA binding buffer) (10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 150 mM KCl), respectively. Unbound proteins were removed with 1 ml RBB/0.5% Triton X-100 four times. The washed beads containing the fusion protein were resuspended in 350 μl of RBB and incubated with 300 μg of testis total extract. Binding was carried out at 4°C for 1 h followed by a rinse with RBB/0.25% Triton X-100 and a 10-min wash in RBB/0.25% Triton X-100 containing 1 mg/ml heparin. The beads were subsequently rinsed four times in RBB/0.25% Triton X-100, and the bound RNAs were then extracted by a standard procedure. The copurifying RNAs were identified by the differential display technique using the 3′ anchored primers (H-T_{11}A, H-T_{11}C, and H-T_{11}G) and 12 distinct 5′ primers. The 5′ primers used were H-AP_1 (AAGCTTGATTGCC), H-AP_2 (AAGCTTCGACTGT), H-AP_3 (AAGCTTTGGTCAG), H-AP_4 (AAGCTTCT-CAACG), H-AP_5 (AAGCTTAGTAGGC), H-AP_6 (AAGCTTGCACCAT), H-AP_7 (AAGCTTAACGAGG),

H-AP_8 (AAGCTTTTACCGC), DAP_{28} (GTTTTCGCAG), DAP_{29} (GATCCAGTAC), DAP_{30} (GATCACGTAC), and DAP_{31} (GATCTGACAC). The bands representing RNAs specifically bound by mT-STAR were cloned into the pGEM-T vector and sequenced.

Riboprobe generation and RT-PCR. Mouse *Fabp9* full-length cDNA was amplified from mouse testis RNA and cloned into the pGEM-T vector (Promega, USA) to generate pGEM-Fabp9 cDNA. The *Fabp9* full-length cDNA was amplified with primers 5′-GCCCACACTTCATGGTTTTCGG-3′ and 5′-TTTTCGATTAGTGCTTTTATTG-3′. Uniformly DIG (digoxigenin)-labeled probe corresponding to the *Fabp9* cDNA was generated by SP6 RNA polymerase from the pGEM-Fabp9 cDNA plasmid that had been linearized with *Nco*I. The *Fabp9* 5′ UTR (untranslated region) was amplified with 5′-GCCCACACTTCATGGTTTTCGG-3′ and 5′-AAGAAGGGCTCAATCATCGTGC-3′, RNA transcript corresponding to the 5′ UTR of the *Fabp9* cDNA was generated by T7 RNA polymerase from the pGEM-5′ UTR cDNA plasmid linearized with *Sal*I (fragment A). Fragments B and C were generated by SP6 RNA polymerase from the pGEM-*Fabp9* coding region and pGEM-3′ UTR plasmids linearized with *Nco*I from PCR-generated templates (5′-TCTTAGGCACCTGGAAACTGAT-3′/5′-TTCGTAGATCCTAGTGCTGAC-3′ and 5′-AAGGGTGTAGCAGAAGGGTAGA-3′/5′-TTTTCGATTAGTGCTTTTATTG-3′, respectively). Other RNA transcripts were generated through the above-mentioned procedures. The RNAs acquired through the SNAAP technique associated with GST-mT-STAR, GST, and GST-mNΔKH proteins were subjected to RT-PCR with specific primers, respectively. The sequences of oligonucleotide primers used for RT-PCR to detect the specific mRNA are given in Table 1.

Electrophoretic mobility shift assay (EMSA). The specific binding of mT-STAR and Fabp9 mRNA was confirmed by EMSA [16]. Binding reactions were carried out with GST-mT-STAR, GST, or GST-mNΔKH proteins and labeled RNAs in binding buffer A (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 67 mg/ml yeast tRNA, 0.25 mg/ml bovine serum albumin, and 1.5 mg/ml heparin) plus 2% 2-mercaptoethanol in a 20 μl total volume at 37°C for 10 min. Protein–RNA complexes are likely to form at 37°C. All labeled RNAs used in EMSA were pretreated at 90°C for 3 min in order to eliminate secondary structure, and then cooled on ice rapidly. Following the binding reactions, 2 μl of 10× loading buffer was added into the complexes, and the samples were immediately loaded onto a 1.5% agarose gel in 1× TBE buffer (0.09 M Tris base, 0.09 M boric acid, and 2 mM EDTA, pH 8.0). The gel was subjected to electrophoresis on ice until separation was completed. The RNA was then electroblotted to a positively charged nylon membrane and subsequently cross-linked to the membrane by short-wavelength ultraviolet radiation. The labeled RNAs on the membrane were detected by conjugating to anti-DIG–alkaline phosphatase. Subsequently, substrate (CDP-Star; Germany) was added to initiate the chemiluminescence reaction. Finally, the membrane was exposed onto an X-ray film.

RESULTS

Isolation of target mRNAs of mT-STAR using SNAAP technique. The interaction of T-STAR and a variety of proteins associated with spermatogenesis suggests that T-STAR can participate in the regulation of gene expression in germ cells. Further investigations of the targets of the T-STAR might lead to the exploration of the functional importance of T-STAR in spermatogenesis. In this study, we have identified 10 mRNA substrates bound by mT-STAR from mouse testis through the SNAAP technique (Table 2). The detailed processes are as follows: first, the GST-mT-STAR and control proteins bound to GST-

Table 1. RT-PCR amplified primer sequences and product size

Name	Accession number	Forward primer	Reverse primer	Product size
Psma3	NM_011184	TCCGCTGGTTTACATTTCTG	ATTGAACCTATGTCCCCTGG	228
Pold1	NM_011131	CATTTCTCCGCATCACCCTG	CCCACAATGTCAGCATCCAC	163
Usp25	NM_013918	GTCCTTACCAAGCACAACAG	TGGAGGTATCCGAGACTGAG	136
Tssk2	NM_009436	TGGAGAAAGCAAGTACCTAG	GGCTTTGCTACTAAGTGGAA	189
Fabp9	NM_011598	AAATGGCTTGGCAAACAGAC	TTAAATCACTGTCCCTGGGG	184
Crisp2	NM_009420	GACTTAGTTGAGACTGATGG	TTTTCAAAGTTCCAGAGGTG	251
A3galt2	NM_001009819	GGGGTTGAAGGACTTATTGT	AAAAGAAGCACTCAGTAAGG	240
Kif2a	NM_008442	GCTTACTCATCCTTCTGTGC	TGTCACAGACTTTAGTTCCG	205
Dalrd3	NM_026378	AGGGCACAAAGAGCGGGACT	AAGGGAAGGACACTGTTGAA	184
Wdr6	NM_031392	ACTTGGACCTTGGTGGGCAT	CTCCCGAAGATGGCTTTGAC	191

beads were incubated with testis extract; second, unbound proteins and RNAs were rinsed away with RBB; finally, RNAs were isolated and subjected to RT-PCR using differential display analysis, respectively. A representative example of differential display gels is illustrated in Fig. 2.

Messenger RNAs copurifying with GST-mT-STAR, GST-mNΔKH, and GST protein were similarly RT-PCR amplified with distinct primers separately. To identify mRNAs that specifically interact with mT-STAR, the pattern obtained with GST-mT-STAR was compared to that obtained with GST-mNΔKH or GST. As expected, several of the bands were equally associated with two (bands F, I, L, and P) or all three (bands B, D, H, J, K, and M) proteins, indicating that they were nonspecifically bound, and these were not further pursued. In contrast, bands A, C, E, G, N, and O represent the PCR products from mRNAs that can only interact with mT-STAR; they were excised from the acrylamide gel, re-amplified with the same set of primers, cloned, and sequenced. The sequence from bands A, E, G, and O did not yield a match when compared to the GenBank (EMBL) database, and so these mRNAs remain unknown. The 193 and 178 nt sequences obtained from bands C and N were expected. The intervening sequence, when searched against the GenBank database, revealed an exact match to the terminal 184 nt of the *Fabp9* gene and 174 nt of *Tssk2* gene ending at the poly(A) tail, respectively.

Use of total testis extract provides the natural circumstances of competitor proteins, increases the specificity of the protein–mRNA binding, and enables the identification of true mRNA substrates [15]. By now, some RNA-binding proteins have been identified, and they might have conjunct target mRNAs. So we chose GST and GST-mNΔKH proteins as control proteins instead of other RNA-binding proteins.

Target mRNA substrates of mT-STAR were further confirmed by RT-PCR. Although some mRNA substrates of mT-STAR were identified through the SNAAP technique, the technique has its own disadvantages. So we further confirmed the fact that mT-STAR protein could bind to these mRNAs through RT-PCR analysis. The RNA species associated with GST-mT-STAR, GST-mNΔKH, and GST were subjected to RT-PCR with specific primers, respectively. Through this process, we verified that these candidate mRNAs can be bound by mT-STAR, not by control proteins (Fig. 3).

Table 2. Summary of mRNA substrates bound by mT-STAR protein

Name	SNAAP primers	Accession number	Tissue	Function
Psma3	H-T_{11}A, H-AP_4	NM_011184	widespread	proteasome α-subunit type 3, generation of peptides
Pold1	H-T_{11}A, H-AP_2	NM_011131	–"–	transcriptional regulation
Usp25	H-T_{11}G, H-AP_4	NM_013918	highly expressed in testis	protein degradation via the 26S proteasome and regulation of many cellular pathways
Tssk2	H-T_{11}C, H-AP_5	NM_009436	male reproductive tract	testis-specific serine/threonine protein kinase, cell differentiation, protein amino acid phosphorylation, spermatogenesis
Fabp9	H-T_{11}A, H-AP_6	NM_011598	testis	preservation of quality of sperm, direction of germ cell fates, spermatogenesis, and testicular germ cell apoptosis
Crisp2	H-T_{11}C, H-AP_6	NM_009420	male reproductive tract	regulation of Ca^{2+} fluxes observed during sperm capacitation, involvement in gamete interaction
A3galt2	H-T_{11}A, H-AP_5	NM_001009819	widespread	catalysis of the synthesis of a range of glycoconjugates
Kif2a	H-T_{11}A, H-AP_4	NM_008442	–"–	regulation of microtubule dynamics, suppression of collateral branch extension, and promotion of spindle bipolarity
Dalrd3	H-T_{11}G, H-AP_3	NM_026378	–"–	splicing
Wdr6	H-T_{11}C, H-AP_5	NM_031392	–"–	signaling, transcription, and proliferation

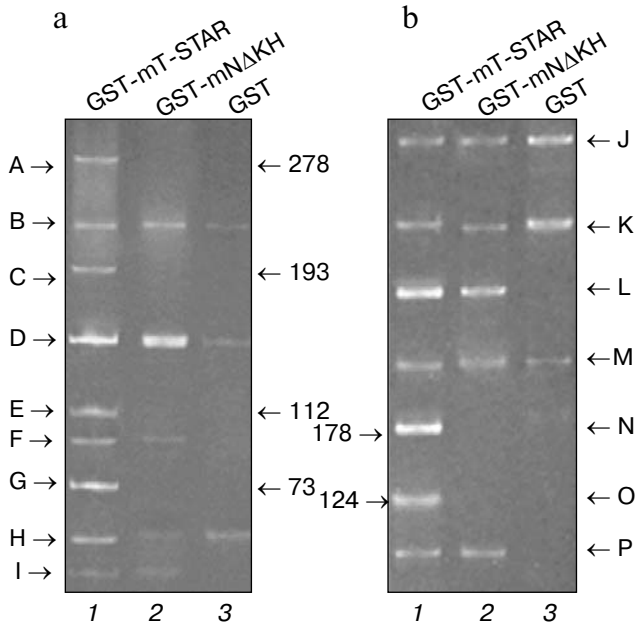

Fig. 2. Identification of specific mRNA substrates through the differential display technique by PAGE analysis. a, b) PCR reactions were carried out using 3′ primer $HT_{11}A$, 5′ primer H-AP_6 and 3′ primer $HT_{11}C$, 5′ primer H-AP_5, respectively. Bands labeled A, C, E, G, N, and O represent PCR products from mRNAs that can only interact with mT-STAR; they were excised from the acrylamide gel, re-amplified with the same set of primers, cloned, and sequenced. In contrast, other bands were PCR products of nonspecific mRNAs.

***Fabp9* mRNA can be bound by mT-STAR protein.** *Fabp9* encodes the most abundant protein of the perinuclear theca of rat spermatozoa, and it is involved in the apoptotic process of spermatogenic cells. Fabp9 is expressed exclusively in the subacrosomal region of the sperm head and is related to the superfamily of lipophilic transport proteins [17, 18]. The testis specificity, reproducible detection as substrate of mT-STAR, and special function in spermatogenesis of *Fabp9* made it an interesting candidate gene to analyze. In our study, the binding specificity of mT-STAR and *Fabp9* was verified by RT-PCR (Fig. 3, lanes *13-15*) and then further confirmed by EMSA (Fig. 4). Instead of the testis total extract that contains both proteins and RNAs, DIG-labeled *Fabp9* was used directly in the binding reaction under the same buffer conditions. Finally, competitive binding assays through EMSA was adopted to strengthen reliability of the consequence (Fig. 5), the contents of competitive binding assay contain mT-STAR, DIG-labeled and free unlabeled *Fabp9* in RBB. Our results showed that the mT-STAR protein could bind to *Fabp9* mRNA, while GST or GST-mNΔKH proteins could not.

Identification of mT-STAR binding site in *Fabp9* mRNA. The binding of *Fabp9* mRNA and mT-STAR was identified through the approach employed above. The binding site was recognized using EMSA as described above, except RNAs consisting of distinct segments of *Fabp9* mRNA were generated *in vitro* and used (Fig. 6a). RNAs corresponding to the 5′ UTR, coding region, and 3′ UTR of the *Fabp9* cDNA were transcribed in the presence of DIG-UTP *in vitro*. The labeled RNAs were subsequently incubated with GST-mT-STAR protein and control proteins in RBB separately. The binding reaction was direct, because recombinant protein was used in the absence of testis extract. The results showed that the sequences bound by mT-STAR were not in the 5′ UTR (Fig. 6b, fragment A) but in the coding region and 3′ UTR of *Fabp9* mRNA (Fig. 6b, fragments B and C). The specific sequences bound by mT-STAR within the coding

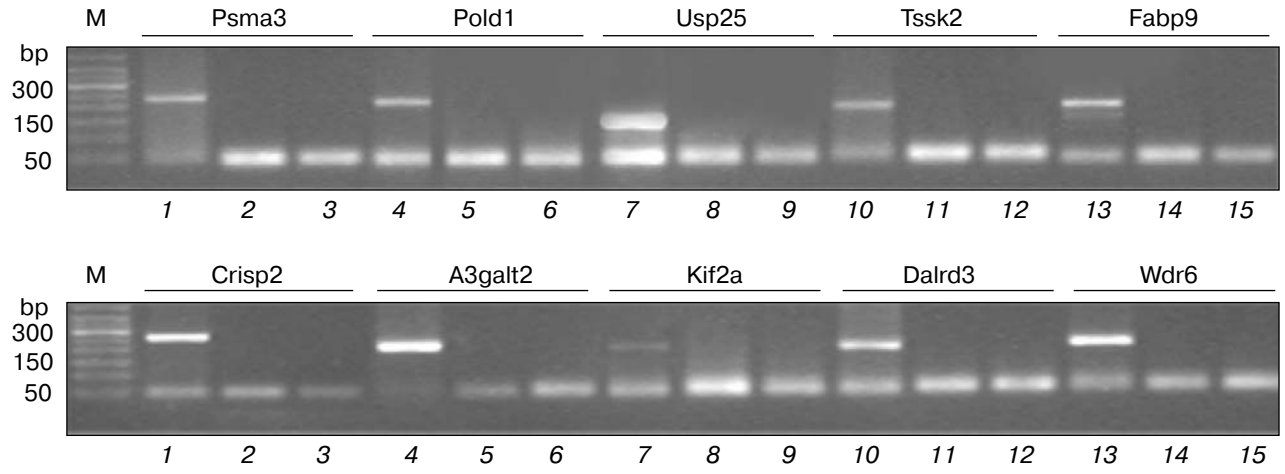

Fig. 3. Target genes of mT-STAR confirmed by RT-PCR. Mouse testis mRNAs bound by GST-mT-STAR (mT-STAR) or the two negative controls were reverse transcribed with specific primers, respectively. Amplification of mRNA bound by mT-STAR, GST, and mNΔKH are shown as following: mT-STAR (lanes *1*, *4*, *7*, *10*, *13*), GST (lanes *2*, *5*, *8*, *11*, *14*), and mNΔKH (lanes *3*, *6*, *9*, *12*, *15*).

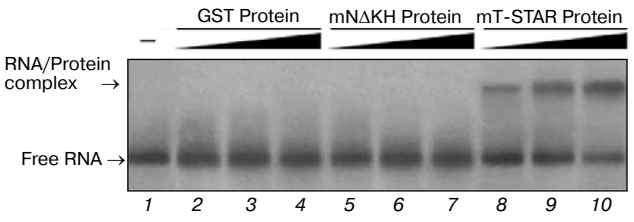

Fig. 4. *Fabp9* mRNA bound by mT-STAR confirmed through EMSA. DIG-labeled *Fabp9* mRNA was incubated with increasing amounts of purified proteins: 100, 200, 400 nM GST protein (lanes *2-4*, respectively); 100, 200, 400 nM GST-mNΔKH (lanes *5-7*, respectively), and 100, 200, 400 nM GST-mT-STAR protein (lanes *8-10*, respectively). Lane *1* contains free RNA only.

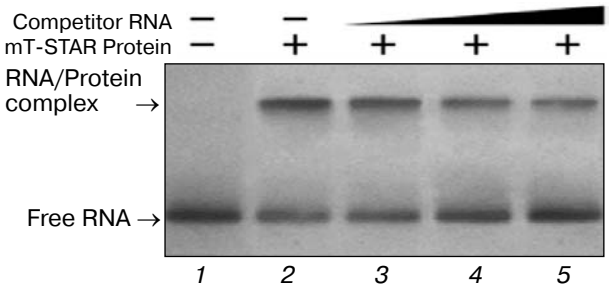

Fig. 5. Competitive RNA-binding assay carried out in the presence of unlabeled *Fabp9* mRNA. About 300 nM mT-STAR proteins (lanes *2-5*) were incubated with 100 fmol DIG-Fabp9 mRNA. Lanes *1-5* contain 0, 0, 500, 1000, and 3000 fmol of unlabeled competitor *Fabp9* mRNA, respectively.

region and 3′ UTR were further identified using smaller labeled segments D, E, F, G, H, I, and J. The results showed that the mT-STAR binding domains were in the 364-396 and 501-587 nt of *Fabp9* mRNAs.

DISCUSSION

T-STAR, an RNA binding protein, has multiple functions in pre-mRNA splicing, signaling, and cell cycle control [9, 19]. These processes might be very finely regulated during spermatogenesis. In this study, we isolated 10 target mRNA substrates by means of the SNAAP technique for mT-STAR. Some target genes, e.g. *Tssk2*, *Crisp2*, and *Fabp9* play important roles in transcriptional regulation, cell differentiation, cell apoptosis, and signal transduction during spermatogenesis.

The *Tssk2* gene encodes a testis-specific serine/threonine kinase that plays a key role during the post-meiotic phase of spermatogenesis through autophosphorylation and phosphorylation of TSKS [20, 21]. Another gene, *Crisp2* encodes a testis-specific protein. The function of Crisp2 is to mediate the binding of spermatogenic cells to Sertoli cells during spermatogenic differentiation [22] and to regulate ion channel activity. Some studies also provide compelling evidence for a role for Crisp2 in the regulation of Ca^{2+} fluxes observed during sperm capacitation [23]. The binding of mT-STAR to *Tssk2* and *Crisp2* mRNAs could affect maturation, cell cycle control, and the fate of sperm cells. Additionally, some other genes selected by SNAAP in this work are expressed widely, such as *Wdr6* and *USP25*. Wdr6 is a novel protein and probably belongs to a highly conserved subfamily of WD-repeat proteins, which are found in all eukaryotes and play an important role in the regulation of a wide variety of cellular functions such as signal transduction, transcription, and proliferation [24], but its function remains unclear during spermatogenesis and needs further study.

Alternative splicing is an important regulatory mechanism that provides a versatile means of increasing the diversity of proteins transcribed from a single pre-mRNA molecule. This is particularly important in the testis because germ cell expansion and differentiation require many cellular changes and regulatory steps [25, 26]. T-STAR is an alternative splicing factor, and it can affect pre-mRNA splicing of transcripts from *CD44* exon V5, transformer-2β, and τ-minigenes in rats [9]. As a candidate target mRNA of mT-STAR, *Crisp2* has four alternative spliceoforms (a, NMp003296; b, AY289796; c, AY292862; d, AY292863), three of which were novel and one (spliceoform c), encoding a longer predicted protein product, had a longer exon 8. All four *Crisp2* spliceoforms were expressed in adult testis and spermatozoa [27]. In addition, the previously known alternatively spliced form of *Crisp2* (a) was located mostly in the testis, and the mouse homolog was identified in round spermatids [28]. These observations suggest that all forms of *Crisp2* could be located in germ cells and highly related to spermatogenesis. All the above suggest that mT-STAR can affect the alternative splicing of *Crisp2* during spermatogenesis.

Among selected target genes, we focused on mouse *Fabp9*. Previous studies have shown that *Fabp9* is involved in testicular germ cell apoptosis and the regulation of the fate of germ cells during spermatogenesis [18, 29]. The mT-STAR protein is expressed at a low level in the early stages of spermatogenesis (spermatogonia and early pachytene spermatocytes), but it could be detected in mid-pachytene nuclei, peaked at late pachytene, and decreased in late round spermatids [11]. *Fabp9* mRNA appears in mid-pachytene spermatocytes, increases in round spermatids, and declines sharply as spermatids elongate [17]. So, the temporospatial expression pattern of mT-STAR protein is consistent with those of *Fabp9* mRNA; this suggests that mT-STAR can regulate the metabolism of *Fabp9* mRNA.

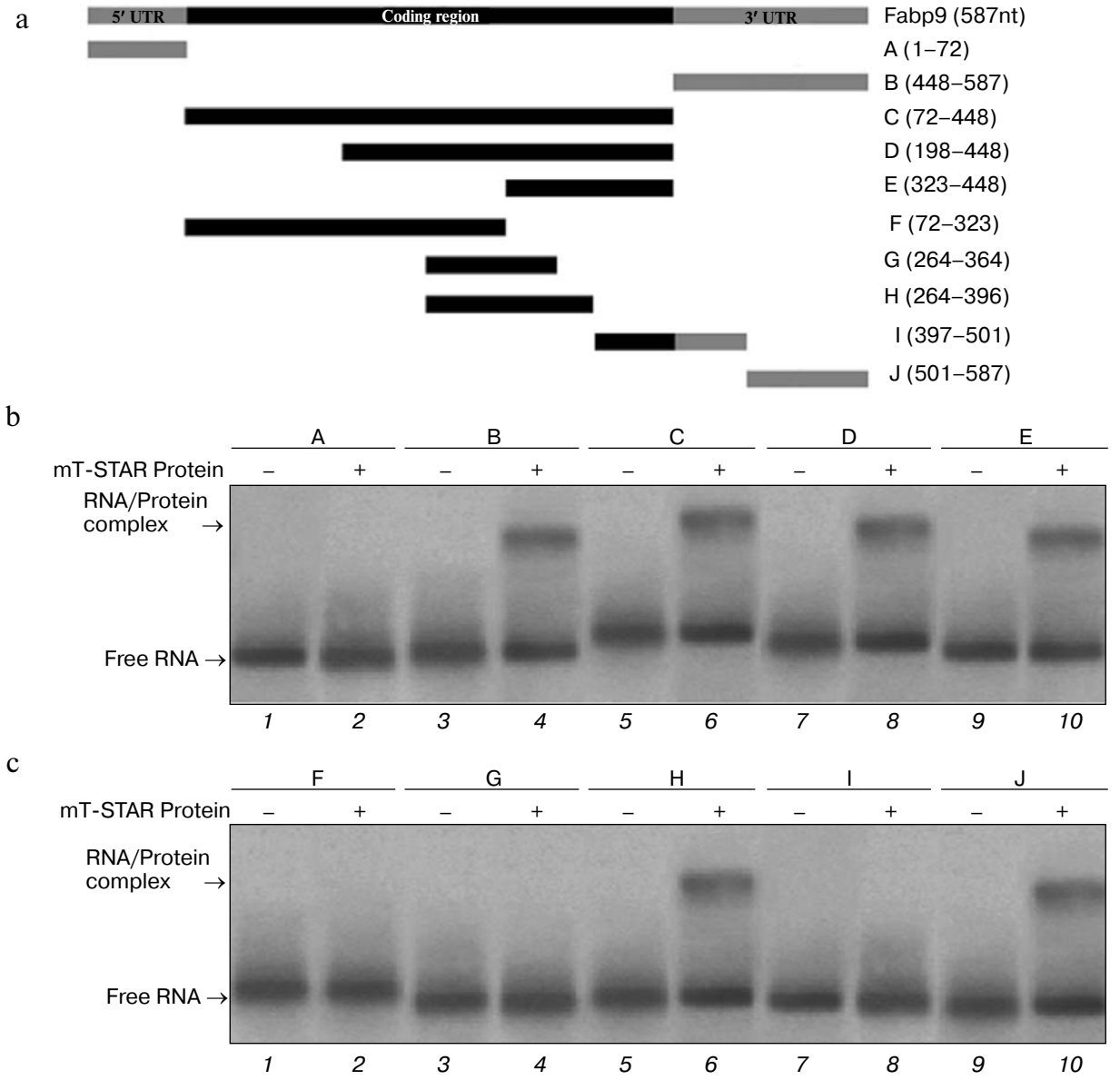

Fig. 6. Identification of mT-STAR-binding sites in *Fabp9* mRNA. a) *Fabp9* mRNA divided into 10 small fragments. The fragments are named A, B, C, D, E, F, G, H, I, and J, respectively. b, c) Various fragments derived from full-length *Fabp9* cDNA were subcloned into pGEM-T vector, and about 100 fmol DIG-labeled RNAs probes bound by mT-STAR at a final concentration of 300 nM were carried out by EMSA, respectively. GST-mNΔKH as a negative control protein.

In this study, we confirmed that mT-STAR could bind to *Fabp9* mRNA, and one of the binding sites is the 364-396 nt region of *Fabp9* mRNA. Previous study has shown that T-STAR participates in the processing of a target gene [9]. The first binding region in *Fabp9* mRNA is localized close to the third exon/intron junction (the last nucleotide of the 3rd exon from the beginning of cDNA sequence is G numbering 406), so we supposed that mT-STAR protein might participate in the splicing of *Fabp9* mRNA. Another binding site is in the 501-587 nt region, which suggests that mT-STAR can affect the translational regulation of *Fabp9* during spermatogenesis. As T-STAR proteins are highly evolutionarily conserved in human, mouse and rat, they might have homologous mRNA targets. Therefore, further study is also needed to confirm whether these RNA substrates are evolutionarily conserved in mammals and whether they are functionally relevant.

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REFERENCES

1. Hecht, N. B. (1998) *Bioessays*, **20**, 555-561.
2. Venables, J. P., and Eperon, I. (1999) *Curr. Opin. Genet. Dev.*, **9**, 346-354.
3. Lee, J., Hong, J., Kim, E., Kim, K., Kim, S. W., Krishnamurthy, H., Chung, S. S., Wolgemuth, D. J., and Rhee, K. (2004) *Mol. Hum. Reprod.*, **10**, 259-264.
4. Elliott, D. J., Millar, M. R., Oghene, K., Ross, A., Kiesewetter, F., Pryor, J., McIntyre, M., Hargreave, T. B., Saunders, P. T., Vogt, P. H., Chandley, A. C., and Cooke, H. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 3848-3853.
5. Weighardt, F., Biamonti, G., and Riva, S. (1996) *Bioessays*, **18**, 747-756.
6. Kamma, H., Portman, D. S., and Dreyfuss, G. (1995) *Exp. Cell Res.*, **221**, 187-196.
7. Elliott, D. J. (2004) *Int. J. Androl.*, **27**, 328-334.
8. Elliott, D. J., Venables, J. P., Newton, C. S., Lawson, D., Boyle, S., Eperon, I. C., and Cooke, H. J. (2000) *Hum. Mol. Genet.*, **9**, 2117-2124.
9. Stoss, O., Olbrich, M., Hartmann, A. M., Konig, H., Memmott, J., Andreadis, A., and Stamm, S. (2001) *J. Biol. Chem.*, **276**, 8665-8673.
10. Vernet, C., and Artzt, K. (1997) *Trends Genet.*, **13**, 479-484.
11. Venables, J. P., Vernet, C., Chew, S. L., Elliott, D. J., Cowmeadow, R. B., Wu, J., Cooke, H. J., Artzt, K., and Eperon, I. C. (1999) *Hum. Mol. Genet.*, **8**, 959-969.
12. Elliott, D. J., Bourgeois, C. F., Klink, A., Stevenin, J., and Cooke, H. J. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 5717-5722.
13. Venables, J. P., Elliott, D. J., Makarova, O. V., Makarov, E. M., Cooke, H. J., and Eperon, I. C. (2000) *Hum. Mol. Genet.*, **9**, 685-694.
14. Paronetto, M. P., Achsel, T., Massiello, A., Chalfant, C. E., and Sette, C. (2007) *J. Cell Biol.*, **176**, 929-939.
15. Trifillis, P., Day, N., and Kiledjian, M. (1999) *RNA*, **5**, 1071-1082.
16. King, P. H. (2000) *Nucleic Acids Res.*, **28**, E20.
17. Pouresmaeili, F., Morales, C. R., and Oko, R. (1997) *Biol. Reprod.*, **57**, 655-659.
18. Kogami, T., Miki, Y., Yamada, T., Umegaki, T., Nishimura, M., Amo, T., Kosaka, J., and Sasaki, J. (2006) *Acta Histochem. Cytochem.*, **39**, 183-192.
19. Kool, J., van Zaane, W., van der Eb, A., and Terleth, C. (2001) *Cell Growth Differ.*, **12**, 535-541.
20. Hao, Z., Jha, K. N., Kim, Y. H., Vemuganti, S., Westbrook, V. A., Chertihin, O., Markgraf, K., Flickinger, C. J., Coppola, M., Herr, J. C., and Visconti, P. E. (2004) *Mol. Hum. Reprod.*, **10**, 433-444.
21. Kueng, P., Nikolova, Z., Djonov, V., Hemphill, A., Rohrbach, V., Boehlen, D., Zuercher, G., Andres, A. C., and Ziemiecki, A. (1997) *J. Cell Biol.*, **139**, 1851-1859.
22. Maeda, T., Nishida, J., and Nakanishi, Y. (1999) *Dev. Growth Differ.*, **41**, 715-722.
23. Gibbs, G. M., Scanlon, M. J., Swarbrick, J., Curtis, S., Gallant, E., Dulhunty, A. F., and O'Bryan, M. K. (2006) *J. Biol. Chem.*, **281**, 4156-4163.
24. Li, D., Burch, P., Gonzalez, O., Kashork, C., Shaffer, L., Bachinski, L., and Roberts, R. (2000) *Biochem. Biophys. Res. Commun.*, **274**, 117-123.
25. Maniatis, T., and Tasic, B. (2002) *Nature*, **418**, 236-243.
26. Boue, S., Letunic, I., and Bork, P. (2003) *Bioessays*, **25**, 1031-1034.
27. Huang, X., Li, J., Lu, L., Xu, M., Xiao, J., Yin, L., Zhu, H., Zhou, Z., and Sha, J. (2005) *J. Androl.*, **26**, 189-196.
28. Kratzschmar, J., Haendler, B., Eberspaecher, U., Roosterman, D., Donner, P., and Schleuning, W. (1996) *Eur. J. Biochem.*, **236**, 827-836.
29. Kido, T., Arata, S., Suzuki, R., Hosono, T., Nakanishi, Y., Miyazaki, J., Saito, I., Kuroki, T., and Shioda, S. (2005) *Dev. Growth Differ.*, **47**, 15-24.