Differential Contribution of the MTOR and MNK Pathways to the Regulation of mRNA Translation in Meiotic and Postmeiotic Mouse Male Germ Cells[1]

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ABSTRACT

Translation of stored mRNAs accounts for protein synthesis during the transcriptionally inactive stages of spermatogenesis. A key step in mRNA translation is the assembly of the initiation complex EIF4F, which is regulated by the MTOR (mammalian target of rapamycin) and MNK1/2 (MAP kinase-interacting kinase 1 and 2) pathways. We investigated the expression and activity of regulatory proteins of these pathways in male germ cells at different stages of differentiation. All translation factors analyzed were expressed in germ cells throughout spermatogenesis. However, while EIF4G and PABP1 (poly[A]-binding protein 1) were more abundant in postmeiotic cells, MTOR and its target EIF4EBP1 (4E-BP1) decreased steadily during spermatogenesis. In vivo labeling showed that pachytene spermatocytes display higher rates of protein synthesis, which are partially dependent on MTOR and MNK activity. By contrast, haploid spermatids are characterized by lower levels of protein synthesis, which are independent of the activity of these pathways. Accordingly, MTOR and MNK activity enhanced formation of the EIF4F complex in pachytene spermatocytes but not in round spermatids. Moreover, external cues differentially modulated the activity of these pathways in meiotic and haploid cells. Heat shock decreased MTOR and MNK activity in pachytene spermatocytes, whereas round spermatids were much less sensitive. On the other hand, treatment with the phosphatase inhibitor okadaic acid activated MTOR and MNK in both cell types. These results indicate that translational regulation is differentially dependent on the MTOR and MNK pathways in mouse spermatocytes and spermatids and suggest that the late stages of germ cell differentiation display constitutive assembly of the translation initiation complex.

gamete biology, kinases, meiosis, MNK, mRNA translation, mRNA translational regulation, MTOR, RNA metabolism, signal transduction, spermatogenesis

INTRODUCTION

Spermatogenesis is a dynamic process that endures for most of the adult life. Shortly after birth in the mouse, the spermatogonial stem cell population resumes the mitotic divisions to maintain itself and to give rise to differentiating spermatogonia [1]. After a defined number of cell divisions, spermatogonia enter meiosis as primary spermatocytes, which undergo two consecutive divisions and ultimately yield haploid spermatids. Meiosis is followed by spermiogenesis, a differentiation phase during which round spermatids elongate and acquire the shape of mature spermatozoa.

Transcriptional control and translational control of gene expression are tightly regulated during spermatogenesis [2–4]. RNA synthesis is abundant in proliferating spermatogonia, middle-late pachytene spermatocytes, and early round spermatids, whereas it is strongly reduced in early meiotic prophase (from leptotene to early pachytene) and in elongating spermatids. In early meiosis, transcription is repressed because the genome is engaged in homologous recombination [4]. During spermiogenesis, histones are replaced by protamines, leading to chromatin condensation and global repression of RNA synthesis [5]. As a consequence, transcription and translation of mRNAs become temporally uncoupled during spermatogenesis [2–7]. In particular, translation of most mRNAs is inhibited in meiotic and haploid male germ cells through formation of ribonucleoprotein particles (RNPs) that globally repress mRNAs. For instance, while spermatogonia readily utilize new mRNAs like proliferating somatic cells, pachytene spermatocytes and round spermatids store most transcripts for several days before recruitment for translation [6–10]. In this regard, it has been shown that 742 transcripts show a dramatic shift between RNPs and polysomes in extracts from prepuberal and adult mouse testes [11]. One subgroup of genes is transcribed during meiosis and translated in round spermatids, whereas another one is translationally activated during spermatid elongation. Although the mechanism of this regulation is still largely unknown, RNA-binding proteins (RBPs) such as YBX2 (Msy2) [12, 13], DAZL [14, 15], and KHDRBS1 (Sam68) [16–18] were shown to promote polysomal recruitment of specific mRNAs and to ensure correct progression of spermatogenesis [3].

A key step in translational regulation occurs at initiation. The 5' end of all nuclear-transcribed mRNAs possesses a cap structure (m7GpppN) that is specifically recognized by the eukaryotic translation initiation factor 4E (EIF4E) [19, 20]. Translation initiation requires association of EIF4E with the scaffold protein EIF4G and the helicase EIF4A to form the translation initiation complex EIF4F. This allows recruitment of PABP1 (poly[A]-binding protein 1), which is bound to the polyadenylated tail of the mRNA, by EIF4G and circularization of the transcript, thereby facilitating translation initiation [19, 20]. The 40S ribosomal subunit is then recruited, in

association with several other initiation factors, and secondary structures in the 5′ untranslated region of the mRNA are involved in the search for the AUG start codon. When the AUG is recognized, the large ribosomal subunit is assembled, and translation begins [19, 20]. Assembly of EIF4F is regulated by members of the EIF4E-binding proteins (4E-BPs), a family of translational repressors [21–23]. They compete with EIF4G for an overlapping binding site on EIF4E in such a way that binding of 4E-BPs and EIF4G is mutually exclusive [22]. The assembly of EIF4F on the mRNA 5′ cap is tightly regulated by signal transduction pathways converging on MTOR (mammalian target of rapamycin) and MNK1/2 (MAP kinase-interacting kinase 1 and 2) [23–25]. These serine-threonine kinases phosphorylate several EIF4F components, as well as ribosomal components. The main substrates of MTOR are the ribosomal protein S6 (rpS6) kinase (RPS6KB1, also known as S6K) and 4E-BPs. Activation of MTOR stimulates protein synthesis through phosphorylation and inactivation of 4E-BPs, thereby causing their release from EIF4E and recruitment of EIF4G [23, 24]. On the other hand, MNKs phosphorylate EIF4E and other RBPs involved in cytoplasmic utilization of mRNAs [25]. MTOR and MNKs sustain proliferation and are often upregulated in cancer cells [23–25]. Thus, although they convey positive signals for cell growth and survival, these pathways need to be tightly controlled to avoid aberrant proliferation and neoplastic transformation.

Despite the crucial role of the MTOR and MNK pathways in the regulation of mRNA translation, their activity and role in male germ cells are still unknown. Thus, because translational regulation has a crucial role in the control of gene expression during spermatogenesis, we set out to investigate these pathways in male germ cells.

MATERIALS AND METHODS

Cell Isolation, Culture, and Treatment

Mice were bred and killed according to procedures approved by European law. Testes of adult CD1 mice (Charles River Italia) were used to prepare pachytene spermatocytes, round spermatids, and elongated spermatids. After dissection of the albuginea membrane, testes were digested for 15 min in 0.25% (w/v) collagenase (type IX; Sigma) at room temperature under constant shaking. Digestion was followed by two washes in minimum essential medium (MEM); hence, seminiferous tubules were digested in MEM containing 1 mg/ml of trypsin (Sigma) for 30 min at 30°C. Digestion was stopped by adding 10% fetal calf serum, and the released germ cells were collected after sedimentation (5 min at room temperature) of tissue debris. Germ cells were centrifuged for 10 min at 1500 rpm at 4°C, and the cellular pellet was resuspended in 20 ml of elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO$_3$, 1.2 mM KH2PO$_4$, 1.2 mM MgSO$_4$[7H$_2$O], 1.3 mM CaCl$_2$, and 11 mM glucose, supplemented with nonessential amino acid [Life Technologies, Inc.], penicillin, streptomycin, and 0.5% bovine serum albumin). Pachytene spermatocyte (fraction 5), round spermatid (fraction 3), and elongated spermatids (fractions 1 and 2) were obtained by elutriation of the unfractionated single-cell suspension as described previously [26]. Homogeneity of cell populations ranged between 80% to 85% (pachytene spermatocytes) and 95% (round spermatids) and was routinely monitored morphologically [27]. Fraction 4 of the elutriation contained a mixed population of early meiotic cells (leptotene, zygotene, and early pachytene spermatocytes) and secondary spermatocytes [16, 26]. Spermatogonia and Sertoli cells were obtained from 8-day-old and 17-day-old mice, respectively, as described previously [28]. After elutriation, pachytene spermatocytes, round spermatids, and elongated spermatids were cultured in MEM, supplemented with 0.5% bovine serum albumin, 1 mM sodium pyruvate, and 2 mM sodium lactate, at a density of 10^6 cells/ml at 32°C in a humidified atmosphere containing 95% air and 5% CO$_2$. After 30 min, cells were treated with 50 nM rapamycin or with 10 µM CGP57380 (an MNK inhibitor) for 2 h. For okadaic acid (OA) treatment, germ cells were treated for 1 or 2 h with 0.5 µM OA (Calbiochem). At the end of the incubation, cells were harvested and washed twice with ice-cold PBS, and protein was extracted as described herein.

Metabolic Labeling of Germ Cells

For the in vivo labeling experiments, 1×10^6 spermatocytes or 4×10^6 round spermatids were incubated in the presence or absence of inhibitors as already indicated. In the last hour of incubation, [^{35}S] cell labeling mix (PRO-MIX [>1000 Ci/mmol]; Amersham) was added to a final concentration of 20 µCi/ml. Cells were lysed in PBS-SDS buffer (150 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, 1.4 mm KH$_2$PO$_4$, and 0.1% sodium dodecyl sulfate), and proteins were precipitated in 10% trichloroacetic acid (TCA). After two washes in 5% cold TCA, insoluble material was collected on GFC filters (Whatman), and the incorporated radioactivity was measured in scintillation fluid.

Preparation of Cell Extracts and Western Blot Analysis

Spermatocytes, round spermatids, and elongated spermatids were resuspended in lysis buffer containing 100 mM NaCl, 10 mM MgCl$_2$, 30 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 10 mM glycerophosphate, 0.5 mM Na$_3$VO$_4$, 1% Triton X-100, and protease inhibitor cocktail (Sigma). Extracts were homogenized, incubated 10 min on ice, and centrifuged for 10 min at 12 000 $\times$ g at 4°C. Protein concentration was determined using Bradford reagent (BioRad). Cell extracts were used for Western blot analysis with the following primary antibodies (1:1000 dilutions): rabbit anti-eIF4E, rabbit anti-mTOR, rabbit anti-4E-BP1, rabbit anti-p-4E-BP1, rabbit anti-eIF4G, and rabbit anti-rpS6 (Cell Signaling Technology); rabbit anti-pSer240/244 rpS6 and rabbit anti-pSer209 eIF4E (BioSource International, Inc.); and mouse anti-tubulin (Sigma). After incubation with secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham), immunostained bands were detected by the chemiluminescent method (Santa Cruz Biotechnology).

Separation of Polysomes and Extraction of Proteins

Spermatocytes, round spermatids, and elongated spermatids were homogenized in lysis buffer (100 mM NaCl, 10 mM MgCl$_2$, 30 mM Tris-HCl [pH 7.5], 1 mM DTT, 30 U/ml of RNasin, and 0.5% Triton X-100). After 5 min of incubation on ice, lysates were centrifuged for 10 min at 12 000 $\times$ g at 4°C. Supernatant fractions were loaded on 15%–50% (wt/vol) sucrose gradients and sedimented by centrifugation for 110 min at 37 000 rpm in a Beckman SW41 rotor (Beckman Coulter). Each gradient was collected in 10 fractions. Proteins were precipitated overnight at −20°C after addition of 6 volumes of a mixture containing 50% ethanol, 25% acetic acid, and 25% methanol and analyzed by Western blot.

7-Methyl-GTP-Sepharose Chromatography

For isolation of EIF4F complex and associated proteins, germ cells were resuspended in lysis buffer containing 50 mM Hepes [pH 7.4], 75 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT, 8 mM ethyleneglycoltetracetic acid, 10 mM glycerophosphate, 0.5 mM Na$_3$VO$_4$, 0.5% Triton X-100, protease inhibitor cocktail, and 30 U/ml of RNasin. Cell extracts were incubated for 10 min on ice and centrifuged at 12 000 $\times$ g for 10 min at 4°C. The supernatants were precleared for 1 h on Sepharose beads (Sigma). After centrifugation for 1 min at 1000 $\times$ g, supernatants were recovered and incubated for 90 min at 4°C with 7-methyGTP-Sel-pharose (Amersham) under constant shaking. Beads were washed three times with lysis buffer, and absorbed proteins were eluted in SDS-PAGE sample buffer.

Immunohistochemistry

Testes were fixed in 4% paraformaldehyde and prepared for immunohistochemical staining as previously described [16]. Testis sections (4 µm) were treated with 0.3% hydrogen peroxide in distilled water for 10 min to block endogenous peroxidase activity. After antigen retrieval [16], slides were incubated with 1:200 dilution of either rabbit anti-pSer240/244 rpS6 or rabbit anti-pSer209 eIF4E (BioSource International, Inc.) for 1 h at room temperature. After washing, antibody detection was accomplished using a biotin-streptavidin horseradish peroxidase detection kit (DAKO EnVision/HRP) according to the manufacturer's instructions.

RESULTS

Analysis of Protein Synthesis in Purified Germ Cells at Different Stages of Differentiation

To investigate the regulation of mRNA translation during spermatogenesis, we initially measured the rate of protein
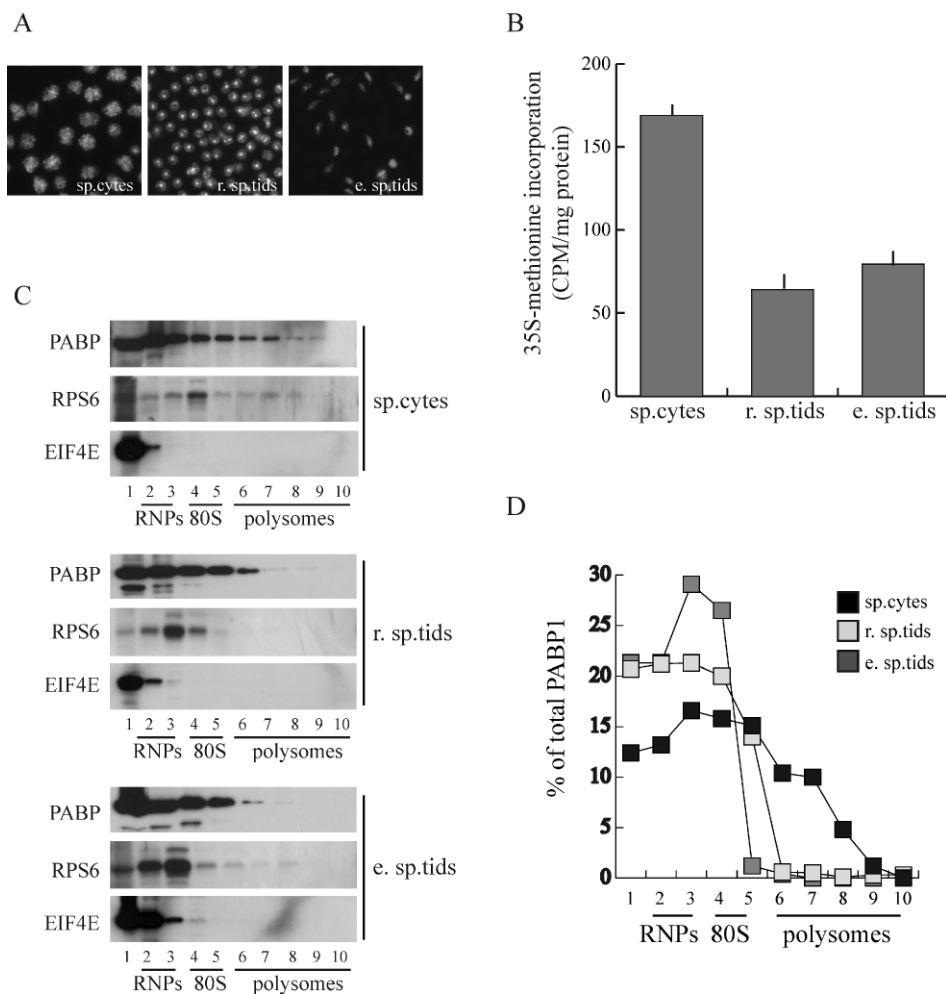
FIG. 1. Analysis of protein synthesis in purified germ cells. **A**) Images of germ cell populations isolated from adult mouse testis (60 dpp) by the elutriation technique after staining nuclei with 1 mg/ml of Hoechst die. Spermatocytes (sp.cytes) and round (r. sp.tids) and elongated (e. sp.tids) spermatids are shown in different panels. Original magnification ×250. **B**) Analysis of the [^{35}S]methionine incorporation into TCA-insoluble proteins. The rate of protein synthesis was expressed as cpm/mg of protein. Data are expressed as the mean ± SD (n = 3). **C**) Polysome fractionation on sucrose gradients obtained from pachytene spermatocytes and round spermatid and elongated spermatid cell extracts. Fraction 1 contains free proteins, fractions 2 and 3 the free RNPs, fractions 4 and 5 the 60S and 80S complexes, and fractions 6–10 the polysomes. Western blot analysis of each fraction from the gradients indicates the distribution of PABP1. Analysis of RPS6 and EIF4E was performed to assess the quality of the fractionation. **D**) Densitometric analysis of the PABP1 signal in each fraction of the Western blot shown in **C**. Results are presented as the percentage of total signal in all fractions.

synthesis. Germ cells were isolated from adult mouse testis (60 days postpartum [dpp]) by elutriation [26] to obtain cell populations enriched in pachytene spermatocytes, round spermatids, and elongated spermatids (Fig. 1A). Germ cells were then cultured as described in *Materials and Methods* before addition of [^{35}S]methionine to the medium in the last hour of incubation. In agreement with previously published data [29], we observed that the rate of protein synthesis was strikingly higher in pachytene spermatocytes than in haploid spermatids (Fig. 1B). Translational activity was also investigated by the distribution of PABP1. This RBP interacts with the poly(A) tail of mRNAs and accompanies them on polysomes during translation [30]. Separation of cell extracts on sucrose gradients showed that more PABP1 associated with the heavy polysomes engaged in translation (fractions 1–5) in extracts from spermatocytes than in extracts from round spermatids and elongated spermatids (Fig. 1, C and D). Although the possibility exists that polysomal profiles are altered by stress imposed during germ cell purification, these experiments indicate that the translational activity of meiotic cells is higher than that of haploid cells.
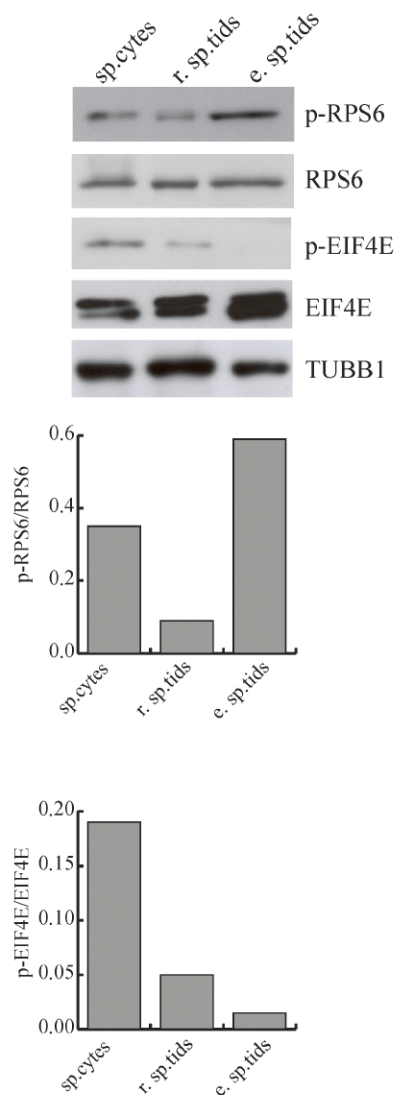
Phosphorylation of RPS6 and EIF4E in Male Germ Cells

In proliferating somatic cells, phosphorylation of RPS6 correlates with activation of the MTOR signal transduction pathway by growth factors [23, 24], whereas phosphorylation of EIF4E by MNKs is observed in response to both proliferation and stress signals [25]. To determine whether the phosphorylation status of these proteins was modified during spermatogenesis, we performed Western blot analyses with phospho-specific antibodies. Densitometric analyses showed that the phosphorylated RPS6:total RPS6 ratio was higher in pachytene spermatocytes than in round spermatids (Fig. 2A), in line with the higher rate of protein synthesis in meiotic cells. However, RPS6 phosphorylation was increased in elongated spermatids (Fig. 2A), even though their translation activity was similar to that of round spermatids. To confirm these results in situ, we performed immunohistochemistry on sections of adult testis. Phosphorylation of RPS6 was detected in pachytene spermatocytes approaching the meiotic divisions (stages XI and XII), and it was stronger in elongated spermatids (stages I–V) (Fig. 2B). By contrast, RPS6 phosphorylation was very low or undetectable in round spermatids and in early spermatocytes (leptotene/zygotene). Thus, phosphorylation of rpS6 increases during the meiotic prophase I, is strongly reduced or abolished after meiosis, and increases steadily during late spermiogenesis.

Phosphorylation of eIF4E showed a simpler pattern during germ cell differentiation. Western blot (Fig. 2A) and immunohistochemistry (Fig. 2B) indicated that EIF4E phosphorylation was detected at almost all stages of the seminiferous tubules, with a peak in pachytene spermatocytes approaching the meiotic divisions and a steady decrease in haploid cells until it became undetectable in the elongated spermatids of tubules in stages I–III.
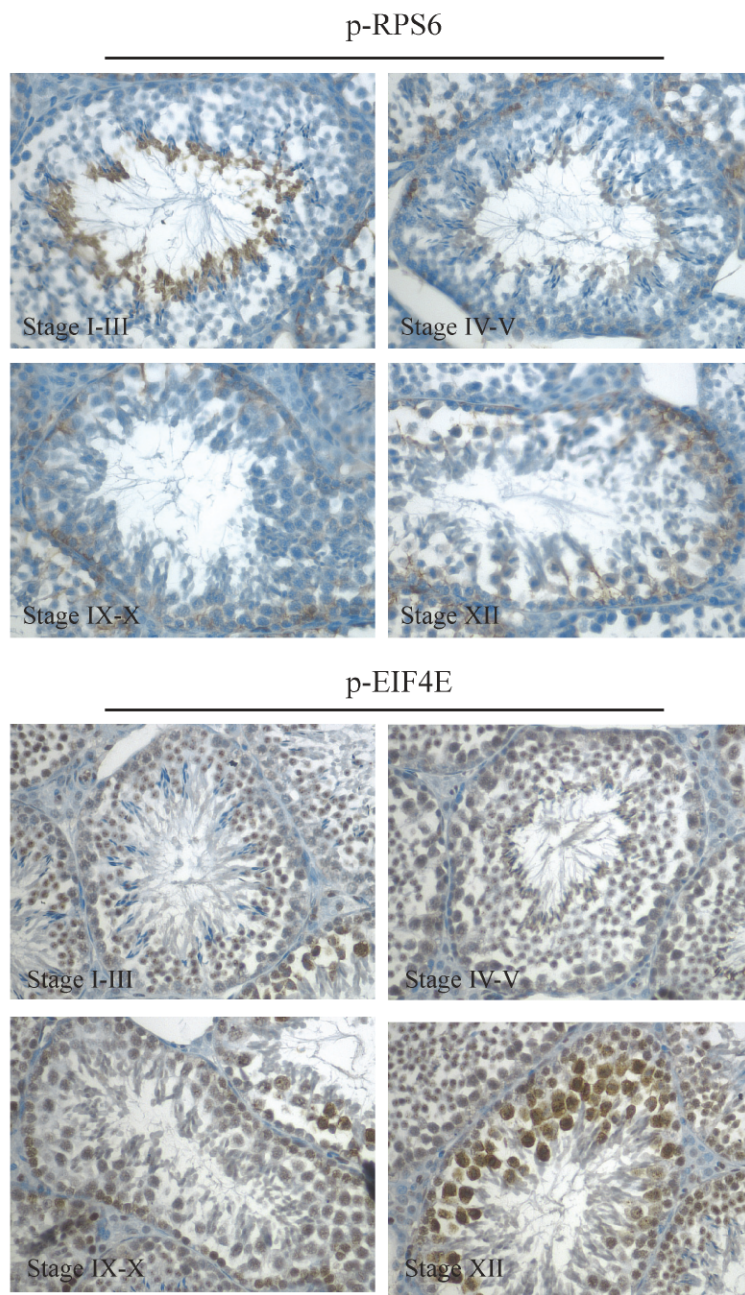
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FIG. 2. Phosphorylation of RPS6 and EIF4E in male germ cells. **A**) Western blot analysis using phospho-specific antibodies in pachytene spermatocytes and round spermatids and elongating spermatids. Bottom panels show the densitometric analysis of the signal in each population. TUBB1 (β-tubulin) was used as loading control. Densitometry was performed using ImageQuant 5.1 software, and results are presented as the ratio between the phosphorylated form and the total form of each protein. **B**) Immunohistochemical analysis of adult mouse testis sections stained with the anti-p-RPS6 (top panels) or the anti-p-EIF4E (bottom) antibodies and counterstained with hematoxylin to detect cell nuclei. The stage of the seminiferous tubule is labeled on the lower left side of each panel. Original magnification ×250.

Analysis of the Expression of Translation Initiation Factors in Male Germ Cells

Next, we analyzed the expression of proteins involved in the MTOR and MNK pathways, which modulate the assembly of the translation initiation complex EIF4F (Fig. 3A) [19, 20]. Although the translation factors analyzed were present in all germ cell types, their expression levels varied substantially (Fig. 3B). MTOR expression decreased progressively during spermatogenesis, with the highest levels in spermatogonia and the lowest levels in round spermatids and elongated spermatids (Fig.

3B). A similar progressive decrease was observed for the MTOR target EIF4EBP1 (4E-BP1), which was almost undetectable in elongated spermatids. By contrast, PABP1 progressively increased during germ cell differentiation, whereas EIF4G sharply increased in postmeiotic germ cells (Fig. 3B). The expression levels of EIF4E did not change substantially, while EIF4A levels were lower in the fraction containing a mixture of early meiotic (leptotene/zygotene) and secondary spermatocytes and were higher in spermatogonia, pachytene spermatocytes, and spermatids (Fig. 3B). Notably, compared with expression in the somatic component of the seminiferous tubule, EIF4E and
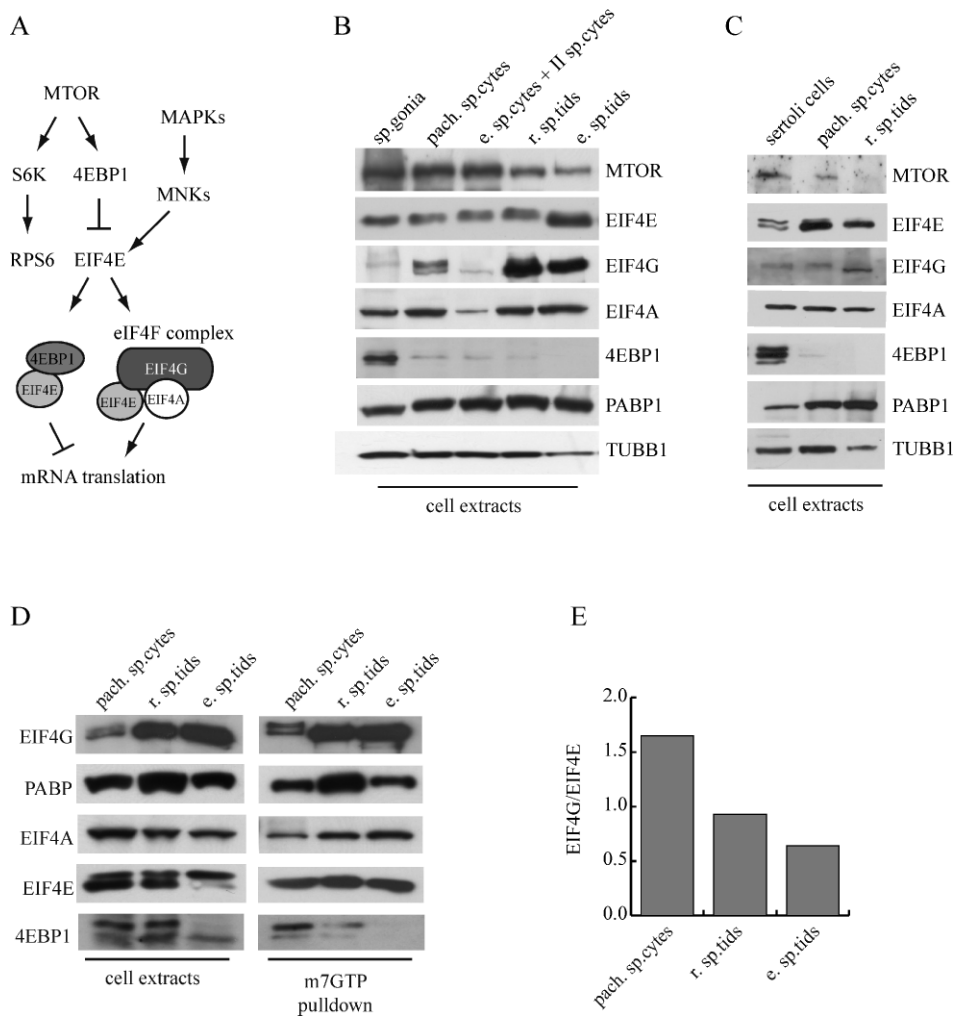
FIG. 3. Expression of translation initiation factors in male germ cells. **A**) Schematic representation of the signal transduction pathways involved in the assembly of the translation initiation complex. **B** and **C**) Western blot analysis of expression of proteins involved in translation initiation in different germ cell types (**B**) or in germ cells and Sertoli cells (**C**). **D**) Western blot analysis of the pull-down assay using 7-methyl-GTP-Sepharose beads to analyze the assembly of the translation initiation complex eIF4F. Spermatocytes, round spermatids, and elongated spermatids isolated from adult mouse testis were harvested after 1 h of culture in MEM medium, and the extracts were processed for binding to 7-methyl-GTP-Sepharose. **E**) Densitometric analysis of the recruitment of EIF4G on the 7-methyl-GTP resin. Values are expressed as the ratio between the signal of EIF4G in the pull-down versus the signal of EIF4G in the cell extract and then normalized for the same ratio calculated for EIF4E:EIF4G (7-methyl-GTP/extract):EIF4E (7-methyl-GTP/extract).

PABP1 were more abundant in meiotic and postmeiotic germ cells than in Sertoli cells, whereas the opposite was observed for EIF4EBP1 and MTOR (Fig. 3C). Expression levels of EIF4A were similar in all cells, whereas EIF4G expression was increased postmeiotically in round spermatids (Fig. 3C).

Regulation of Translation Initiation by the MTOR and MNK Pathways in Male Germ Cells

To investigate the regulation of cap-dependent translational activity in germ cells, we initially analyzed EIF4F assembly by pull-down assay using 7-methyl-GTP-Sepharose beads, which mimic the 5′ cap structure of the mRNA [31]. EIF4E association with EIF4G and EIF4A was notably increased in haploid germ cells compared with pachytene spermatocytes (Fig. 3D). However, the total levels of EIF4G in the extract were sharply higher in postmeiotic cells, suggesting that the increase in EIF4F assembly may simply reflect the higher abundance of this protein with respect to its competitor EIF4EBP1 (Fig. 3, B and D). Indeed, when we calculated the efficiency of EIF4F assembly as the percentage of EIF4G recruited to the cap with respect to the amount present in the extract, the EIF4G:EIF4E ratio was higher in spermatocytes than in round spermatids and elongated spermatids (Fig. 3E). These experiments indicate that, in line with their increased rate of protein synthesis, the efficiency of EIF4F formation is higher in spermatocytes than in round spermatids and elongated spermatids (Fig. 1B).

EIF4F assembly is regulated by the MTOR and MNK pathways through phosphorylation of EIF4EBPs and EIF4E, respectively [23–25]. To study the involvement of these pathways in the regulation of translation in male germ cells, we used the specific inhibitors rapamycin (for MTOR) and CGP57380 (for MNKs) (Fig. 4A) [23–25, 32]. Pachytene spermatocytes, round spermatids, and elongated spermatids were cultured for 3 h in medium, supplemented with 10 nM rapamycin or 10 μM CGP57380, and [^{35}S]methionine was added in the last hour of incubation. Interestingly, inhibition of either MTOR or MNK activity reduced protein synthesis by approximately 20% in pachytene spermatocytes (Fig. 4B), whereas round spermatids and elongated spermatids were completely unaffected by these treatments. These results suggested that the MTOR and MNK pathways regulate EIF4F assembly in meiotic but not haploid germ cells. To test this hypothesis, we performed pull-down assays with 7-methyl-GTP-Sepharose beads. In line with what was observed with the rate of protein synthesis, treatment with both drugs decreased EIF4F formation in primary spermatocytes, as indicated by the reduced binding of EIF4E to EIF4G, EIF4A, and PABP1 (Fig. 5A). The effect of CGP57380 on EIF4F assembly in spermatocytes was unpredicted, as previous experiments in prostate cancer cells showed that inhibition of eIF4E phosphorylation blocked translation at a step downstream of
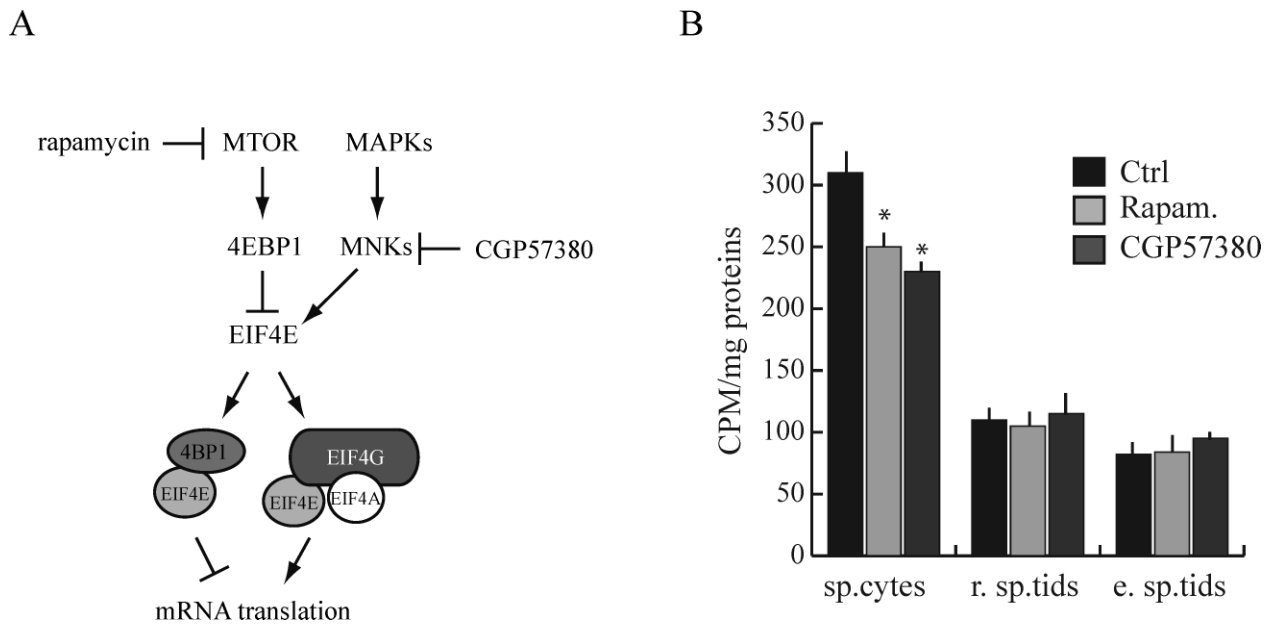
A

B

FIG. 4. Regulation of translation initiation by the MTOR and MNK pathways. **A**) Schematic representation of the MTOR and MAPK pathways involved in the regulation of mRNA translation. The inhibitors (rapamycin and CGP57380) used in this study and their targets are indicated. **B**) Measurement of [^{35}S]methionine incorporation in TCA-insoluble protein pellets of spermatocytes, round spermatids, and elongated spermatids isolated by elutriation technique and cultured in MEM medium in the presence of rapamycin or CGP57380 for 3 h. [^{35}S]methionine was added in the last hour of incubation. Data are presented as described in Figure 1B (n = 3). Statistical analysis was performed by Student t-test. *$P < 0.01$.

EIF4F assembly [32]. Notably, inhibition of MTOR or MNK activity exerted no effects on EIF4F in round spermatids (Fig. 5B), confirming the lack of inhibition of translation observed with metabolic labeling. These results indicate that mRNA translation is differentially dependent on the MTOR and MNK pathways in meiotic and postmeiotic male germ cells under basal culture conditions.

Regulation of the MTOR and MNK Pathways by Heat Shock and OA in Male Germ Cells

Next, we investigated whether the pathways that modulate EIF4F assembly were sensitive to external or internal cues in male germ cells. In the mouse, male germ cells develop at

32°C, and spermatogenesis is severely inhibited by thermal stress. Remarkably, exposure to high temperatures (37–44°C) causes inhibition of protein synthesis in pachytene spermatocytes but not in haploid spermatids [33]. To test whether these differences were due to differential regulation of the MTOR and MNK pathways, we analyzed the impact of thermal stress on RPS6 and EIF4E phosphorylation in germ cells. Raising the temperature to 39°C for 1–4 h caused a decrease in EIF4E and RPS6 phosphorylation in pachytene spermatocytes (Fig. 6A). Phosphorylation of EIF4E was slightly reduced also in haploid cells exposed to higher temperatures, although to a lower extent than in spermatocytes (Fig. 6A). By contrast, RPS6, which was weakly phosphorylated in control cells, was insensitive to heat shock in round spermatids (Fig. 6A). These

FIG. 5. Analysis of EIF4F formation in the presence of MTOR or MNK inhibitors with pull-down assay using 7-methyl-GTP-Se-pharose beads to analyze the assembly of the translation initiation complex EIF4F in the presence or absence of rapamycin or CGP57380. Spermatocytes (**A**) and round spermatids (**B**) isolated from adult mouse testis were cultured for 2 h in medium, supplemented with rapamycin or CGP57380. The extracts were processed for binding to 7-methyl-GTP-Sepharose beads and analyzed by Western blot for the indicated proteins.
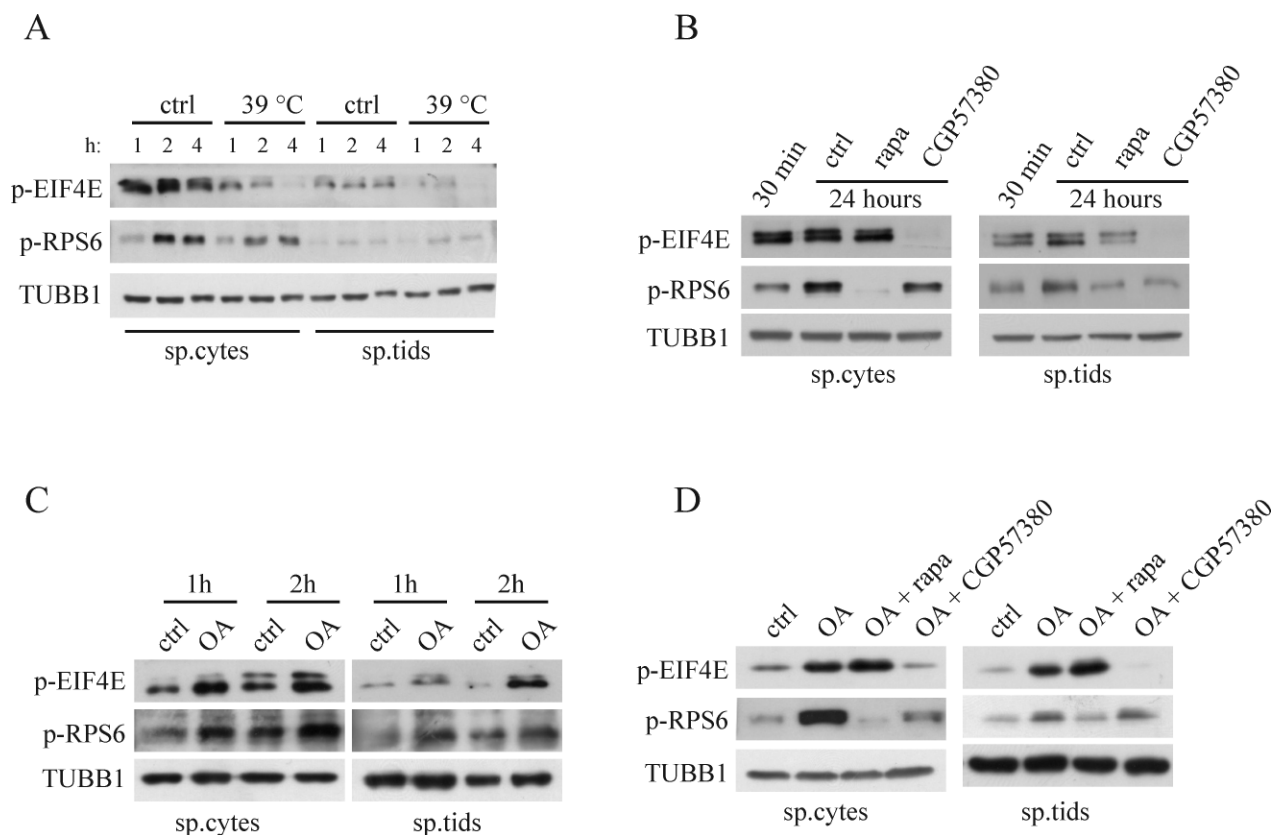
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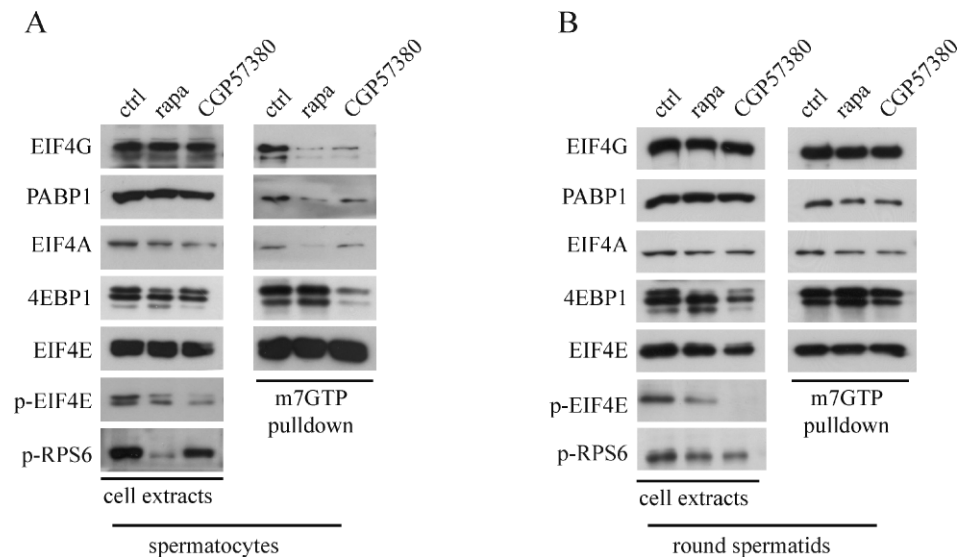
FIG. 6. Regulation of the MTOR and MNK pathways after internal or external cues in male germ cells. **A**) Western blot analysis of RPS6 and EIF4E phosphorylation in spermatocytes and round spermatids after heat shock. Male germ cells were incubated for 1–4 h at 39°C. **B**) Western blot analysis of RPS6 and EIF4E phosphorylation in pachytene spermatocytes and round spermatids cultured in MEM for 24 h in the absence or presence of rapamycin or CGP57380. **C** and **D**) Male germ cells were treated with OA for 1–2 h in the absence (**C**) or presence (**D**) of rapamycin or CGP57380. Phosphorylation of RPS6 and EIF4E was analyzed by Western blot.

results indicate that the MTOR and MNK pathways are differentially regulated by thermal stress in meiotic and postmeiotic male germ cells.

We also tested whether activation of these pathways could be elicited by external cues. Male germ cells are unresponsive to the majority of growth factors tested, likely because their direct contact with bloodstream components is prevented by tight junctions between Sertoli cells within the seminiferous tubule [34, 35]. In line with this notion, we found that addition of serum or epidermal growth factor to the culture for 1–6 h did not exert any effect on the MTOR and MNK pathways (data not shown). However, we observed that phosphorylation of rpS6 was increased in pachytene spermatocytes, and to a lesser extent in round spermatids, cultured in MEM for 24 h (Fig. 6B). This increase in phosphorylation was mediated by MTOR, as demonstrated by the inhibitory effect of rapamycin. On the other hand, activation of EIF4E phosphorylation was more evident in spermatids than in spermatocytes, even though in both cell types inhibition of MNK activity completely suppressed this event (Fig. 6B).

Activation of cell cycle-dependent kinases and mitogen-activated protein kinases can be triggered by exposure of male meiotic germ cells to the phosphatase inhibitor OA [27, 34, 36]. Under this condition, pachytene spermatocytes progress to metaphase I, during which phosphorylation of eIF4E and rpS6 is increased in stage XII tubules (Fig. 2B). We observed that treatment of both pachytene spermatocytes and round spermatids with OA for 1–2 h induced phosphorylation of RPS6 and

EIF4E in culture (Fig. 6C). Moreover, these posttranslational modifications relied on the MTOR and MNK pathways, as treatment of germ cells with rapamycin and CGP57380 abolished OA-induced phosphorylation of RPS6 and EIF4E, respectively (Fig. 6D). These results show that, although differentially active under basal conditions, the MTOR and MNK pathways can be stimulated or repressed by external cues in both meiotic and postmeiotic germ cells.

DISCUSSION

The proper timing of differentiation events that take place in the absence of transcription during spermatogenesis needs fine tuning of mRNA translation [2, 3, 7], the mechanisms of which are largely unknown in male germ cells. Several RBPs are highly expressed in germ cells, and implicated mRNA storage and translation control have been demonstrated in some of them [3]. On the other hand, no information is available about the role of signal transduction pathways that regulate translation initiation in male germ cells.

The aim of this work was to understand the contribution of the MTOR and MNK signal transduction pathways, which are known to regulate translation in somatic cells [23–25], in male germ cells at different developmental stages. We initially measured the rate of [^{35}S]methionine incorporation in meiotic and postmeiotic germ cells, and we observed that spermatocytes display a higher rate of protein synthesis compared with spermatids. In agreement with these data is the higher amount of polysome-associated PABP1 in spermatocytes than in

spermatids, despite its higher expression levels in postmeiotic germ cells. Although other PABPs may contribute to translation activity in spermatids, the distribution of PABP1 fits well with the lower protein synthesis rate of haploid male germ cells. The higher translational activity of spermatocytes is also supported by phosphorylation of rpS6, a well-known marker of active translation [23, 24]. Western blot and immunohistochemical analyses showed that rpS6 phosphorylation was reduced in round spermatids. Thus, our results indicate that pachytene spermatocytes and round spermatids differ in both the rate of translation and the level of phosphorylation of RPS6. However, we also observed an increase of p-RPS6 in the late stages of spermiogenesis (stages V–XI), which did not correlate with the low rates of protein synthesis measured by metabolic labeling, nor with the drastic reduction in MTOR expression. Other kinases (such as p90RSKs) have also been shown to mediate rpS6 phosphorylation [23–25]. These kinases are expressed in postmeiotic germ cells [36], suggesting that rpS6 phosphorylation might become uncoupled from MTOR in late spermiogenesis.

These conclusions are also in agreement with the efficiency of EIF4F complex assembly, which often represents the limiting step in translational control [20]. Indeed, although EIF4E was associated with higher amounts of EIF4G in round spermatids than in pachytene spermatocytes, we also observed that EIF4G was strongly upregulated in haploid cells. When we calculated the efficiency of EIF4F assembly as the ratio between EIF4G bound to 5′ cap-associated (7-methyl-GTP) EIF4E versus its level in the cell extract, spermatocytes displayed higher efficiency (Fig. 3E). Because EIF4F assembly is mainly regulated by the activity of MTOR, we analyzed the expression of this kinase (and of some of its substrates) in germ cells during differentiation. Remarkably, we found that expression of MTOR (and its substrate EIF4EBP1) steadily declined from spermatogonia (which resembled the somatic Sertoli cells in translation factor expression levels) to elongated spermatids. Thus, because EIF4F formation depends on the competition between EIF4G and EIF4EBP1 for the binding to EIF4E [22], the higher amount of EIF4G bound to EIF4E in spermatids is likely a reflection of the altered ratio between these antagonistic factors. Nevertheless, assembly of EIF4F is insufficient to ensure high rates of mRNA translation and protein synthesis in round spermatids in the absence of high MTOR activity.

In somatic cells, the MTOR and MNK pathways regulate protein synthesis [32]. Our [^{35}S]methionine labeling experiments and 7-methyl-GTP-Sepharose pull-down assays demonstrated that inhibition of either of these pathways reduced mRNA translation in pachytene spermatocytes. By contrast, round spermatids and elongated spermatids were completely insensitive to MTOR and MNK inhibitors. These results suggest that the basal activity of these pathways is higher in meiotic cells and support the increased rates of protein synthesis in spermatocytes. Phosphorylation of EIF4E by MNK in germ cells is of particular interest. This event correlates with the proliferation rate of somatic cells, and it is increased in cancer [25, 37]. However, we previously showed that this pathway did not affect formation of EIF4F in cancer cells but rather that it affected postinitiation processes to enhance translation [32]. On the other hand, inhibition of MNK activity in pachytene spermatocytes decreased EIF4F assembly. This effect might be indirect due to cross talk between the MNK and MTOR pathways in these cells. In support of this hypothesis, we observed that CGP57380 reduced phosphorylation of RPS6, a well-known target of the MTOR pathway, in meiotic germ cells.

Although round spermatids were insensitive to MTOR and MNK inhibitors, these pathways are maintained in haploid cells and can be activated by external cues. We found that OA stimulated MTOR-dependent phosphorylation of RPS6- and MNK-dependent phosphorylation of EIF4E in both meiotic and postmeiotic germ cells. In the case of spermatocytes, the effect of OA correlates with what we observed in situ. Indeed, OA is known to induce meiotic progression of spermatocytes from the pachytene stage to metaphase I [27, 34, 36]. In line with this notion, phosphorylation of both RPS6 and EIF4E was increased in stage XII spermatocytes, which are undergoing meiotic divisions. Because the MTOR and MNK pathways respond to stimulation of growth factor receptors and integrin-receptors [23–25], it is possible that specific signals originating at the Sertoli cell-germ cell junctions at this stage of the seminiferous tubule trigger their activation. Notably, although these signals appeared to be absent in haploid spermatids, the MTOR and MNK pathways could be exogenously activated. Thus, these pathways might transiently support mRNA translation in response to specific testicular cues in both spermatocytes and spermatids.

We also found that both MTOR and MNK pathways were inhibited by thermal shock in primary spermatocytes, whereas only mild (with EIF4E) or no (with RPS6) effects on phosphorylation were observed in round spermatids. This result might be due to the different basal activity levels of these pathways in the germ cells, with round spermatids showing very low activity already in control cells. In addition, the different sensitivity of these pathways to thermal shock also correlates with its effect on protein synthesis rates in meiotic and postmeiotic germ cells. It was previously shown that increasing the temperature to 39°C decreased protein synthesis in spermatocytes but not in spermatids [33]. Our results now suggest that the effects on protein synthesis might be a reflection of specific inhibition of the MTOR and MNK pathways in meiotic cells.

In conclusion, our results indicate that the pathways that regulate initiation of mRNA translation are differentially regulated in meiotic and postmeiotic germ cells. In primary spermatocytes, which express a lower EIF4G:EIF4EBP1 ratio, they support EIF4F assembly and protein synthesis, possibly in response to external cues that increase when germ cells approach the meiotic divisions. In round spermatids, decreased expression of the inhibitory EIF4EBP1 is sufficient to ensure formation of EIF4F and the lower levels of protein synthesis that characterize these cells. However, external cues are capable of transiently increasing the activity of the MTOR and MNK pathways also in haploid cells, and we suggest that this might occur in vivo in response to specific somatic cell-germ cell interactions.

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