The MAPK pathway triggers activation of Nek2 during chromosome condensation in mouse spermatocytes

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SUMMARY

Chromosome condensation during the G2/M progression of mouse pachytene spermatocytes induced by the phosphatase inhibitor okadaic acid (OA) requires the activation of the MAPK Erk1. In many cell systems, p90Rsks are the main effectors of Erk1/2 function. We have identified p90Rsk2 as the isoform that is specifically expressed in mouse spermatocytes and have shown that it is activated during the OA-triggered meiotic G2/M progression. By using the MEK inhibitor U0126, we have demonstrated that activation of p90Rsk2 during meiotic progression requires activation of the MAPK pathway. Immunofluorescence analysis indicates that activated Erks and p90Rsk2 are tightly associated with condensed chromosomes during the G2/M transition in meiotic cells. We also found that active p90Rsk2 was able to phosphorylate histone H3 at Ser10 in vitro, but that the activation of the Erk1/p90Rsk2 pathway was not necessary for phosphorylation of H3 in vivo. Furthermore, phosphorylation of H3 was not sufficient to cause condensation of meiotic chromosomes in mouse spermatocytes. Other proteins known to associate with chromatin may represent effectors of Erk1 and p90Rsk2 during chromosome condensation. Nek2 (NIMA-related kinase 2), which associates with chromosomes, plays an active role in chromatin condensation and is stimulated by treatment of pachytene spermatocytes with okadaic acid. We show that inhibition of the MAPK pathway by preincubation of spermatocytes with U0126 suppresses Nek2 activation, and that incubation of spermatocyte cell extracts with activated p90Rsk2 causes stimulation of Nek2 kinase activity. Furthermore, we show that the Nek2 kinase domain is a substrate for p90Rsk2 phosphorylation in vitro. These data establish a connection between the Erk1/p90Rsk2 pathway, Nek2 activation and chromosome condensation during the G2/M transition of the first meiotic prophase.

Key words: Meiosis, Spermatocytes, MAPK, Nek2, Chromosome, Mouse

INTRODUCTION

Sexual reproduction requires the fusion of two haploid cells to form a diploid zygote. In higher eukaryotes, this implies that specialized diploid cells, the germ cells, reduce their chromosome content during meiosis. A single round of DNA replication followed by two cell divisions characterizes this process, such that a single diploid cell gives rise to four haploid cells. In the first division, homologs are initially held together by the synaptonemal complex, which dissolves at the end of the pachytene stage, and subsequently by chiasmata produced during homologous recombination (Roeder, 1997). At anaphase, sister chromatids do not separate and homologous chromosomes segregate to the opposite poles of the spindle into the two daughter cells (reviewed by Roeder, 1997; Handel and Eppig, 1998; Biggins and Murray, 1999). The second meiotic division resembles mitosis and sister chromatids are separated in the daughter cells to give rise to haploid spermatids (Handel and Eppig, 1998).

Most of the knowledge on meiotic cell cycle events derives from studies performed on Xenopus oocytes, which are synchronized at the prophase of the first meiotic division and can be induced to progress through the cycle by stimulation with progesterone (reviewed by Sagata, 1997). The hormonal treatment leads to several biochemical changes that result in maturation of the oocyte: a decrease in cAMP-dependent protein kinase activity; the synthesis of the protein kinase Mos and activation of the MAPK pathway; activation of the Polo-like-kinase (Plx1) pathway; dephosphorylation of Cdc2 by the dual specificity phosphatase Cdc25C; and the consequent activation of the CyclinB/Cdc2 complex, also known as maturation promoting factor (MPF) (Sagata et al., 1988; Sagata et al., 1989a; Sagata et al., 1998b; Qian et al., 1998; Qian et al., 2001). Although MPF activation and oocyte maturation can be obtained in vivo by microinjection of constitutively active forms of MAPKs (Gotoh et al., 1995; Haccard et al., 1995), recent evidence suggests that physiologically activation of the MAPK pathway is not sufficient for progression of oocytes through the first meiotic cycle. Indeed, inhibition of MAPKs by preincubation of oocytes with the specific MEK inhibitor...
U0126 did not block maturation induced by progesterone (Gross et al., 2000). However, Qian and collaborators have demonstrated that immunodepletion of Plx1 from Xenopus oocyte extracts completely prevents activation of Cdc25C and MPF, indicating a crucial role of this kinase in the control of MPF function (Qian et al., 2001).

Maintenance of chromatin condensation between the two meiotic divisions contributes to prevent inappropriate DNA replication and allows progression between two metaphases without the interphase of a normal cell cycle (Gross et al., 2000). Suppression of DNA replication requires the incomplete inactivation of MPF at anaphase of the first meiotic cycle (Furuno et al., 1994). In this regard, both incomplete degradation and new synthesis of cyclin B contribute to maintain MPF activity during the exit from metaphase I and to allow progression to meiosis II without interphase (Kobayashi et al., 1991; Roy et al., 1996; Ohsumi et al., 1994; Gross et al., 2000). A fundamental role in this process is played by the MAPK pathway that, through the concerted action of Mos, Erks and p90Rsk, maintains the inhibition of the anaphase-promoting complex (APC), thereby reducing cyclin B degradation (Gross et al., 2000; Taieb et al., 2001). Although inhibition of Erks activation does not prevent cell cycle progression and activation of MPF, when the MAPK pathway is blocked, the metaphase I spindle cannot form and the complete destruction of cyclin B by the APC allows entry into interphase and DNA duplication between metaphase I and II (Gross et al., 2000). Therefore, even though it is not directly involved in regulation of the activity of cell-cycle dependent kinases, the MAPK pathway plays a crucial role in meiosis by allowing the reduction of the genome to a haploid content. Later in meiosis, Mos and MAPKs are also necessary for the arrest of oocytes at the metaphase of the second meiotic division (Sagata, 1997), and this cytostatic role is mediated by the MAPK effector p90Rsk2 (Gross et al., 1999; Bath and Ferrell, 1999). However, such a role is restricted to female meiosis, as spermatocytes do not arrest at metaphase and the male meiotic cycle is continuous.

Not much is known on meiotic events in mammals. Meiotic resumption in mammalian oocytes is under the hormonal control of intracellular cAMP levels (Handel and Eppig, 1998). A drop in intracellular cAMP triggers meiotic resumption that temporally correlates with activation of both MAPK and MPF. Experiments performed with Mos knockout mice have shown that Mos and the MAPK pathway do not play a role in meiotic resumption but are required as cytostatic factors that arrest the cell cycle at metaphase II in ovulated oocytes (Colledge et al., 1994; Hashimoto et al., 1994). In male mouse spermatocytes, the prophase of the first meiotic division is a slow and continuous process that ensures DNA repair after crossing over and cell growth before the two subsequent divisions that will give rise to four haploid spermatids. It has been reported that mid-pachytene spermatocytes can be induced to complete the prophase of the first meiotic division and enter metaphase by incubation for 4-6 hours with the phosphatase inhibitor okadaic acid (OA) (Wiltshire et al., 1995). OA is able to bypass the steps involved in this progression (Wiltshire et al., 1995; Cobb et al., 1999a). Indeed, metaphase chromosomes obtained by treatment of spermatocytes with OA are normal bivalents in which the synaptonemal complex has dissolved, crossing over has been completed and chiasmata are present (Wiltshire et al., 1995). Spermatocyte G2/M progression is accompanied by activation of MPF and the MAPK Erk1 (Wiltshire et al., 1995; Sette et al., 1999), and it has been shown that inhibition of the MAPK pathway prevents efficient chromosome condensation independently of MPF during transition from prophase to metaphase (Sette et al., 1999).

Pathways that trigger chromosome condensation and alternative to MPF have been described in mitotic cells. In cycling extracts obtained from Xenopus eggs, phosphorylation of histone H3, which correlates with chromatin condensation, is mediated by the Aurora kinases (Murnion et al., 2001; Scrittori et al., 2001). Activation of the NIMA-like kinases (never-in-mitosis in Aspergillus nidulans) Nek1, Nek2 and Nek3 has also been reported to induce chromatin condensation independently of MPF (Lu and Hunter, 1995). This observation is particularly interesting because Nek2 is expressed in mouse meiotic cells and it is associated with condensing chromosomes during the prophase of the first division (Tanaka et al., 1997; Rhee and Wogelmuth, 1997). Furthermore, it has been observed that Nek2 is activated during OA-induced G2/M progression, and that the time course of its activation correlates with that of chromosome condensation (Rhee and Wogelmuth, 1997). Nek2 is known to phosphorylate the centrosome-associated protein C-Nap1 (Fry et al., 1998a) and it may play a role in centrosome separation and chromosome dynamics (Fry et al., 1998b; Uto and Sagato, 2000). However, it is currently non known what mechanisms are involved in activation of Nek2 and what role the kinase plays during meiotic progression of mouse spermatocytes.

In this study, we have investigated the role played by the MAPK pathway in chromosome condensation during the G2/M progression induced by OA in mouse pachytene spermatocytes. We found that p90Rsk2 was the main p90Rsk isoform expressed in these cells and that it was activated by the MAPK pathway during meiotic progression. Inhibition of Erks and p90Rsk2 activation blocks condensation of metaphase chromosomes. Furthermore, activated Erks and p90Rsk2 were found in tight association with the condensed meiotic chromosomes. Although p90Rsk2 is able to phosphorylate histone H3 in vitro, in vivo phosphorylation of the histone induced by OA treatment of spermatocytes was not affected by inhibition of p90Rsk2 activity. Finally, we found that Erks and p90Rsk2 were required for the activation of Nek2 during the G2/M transition in vivo, and that p90Rsk2 is able to activate and phosphorylate Nek2 in vitro. These data suggest that chromosome condensation in meiotic cells requires the activation of the NIMA-like kinase Nek2 by the MAPK pathway.

**MATERIALS AND METHODS**

**Preparation of testicular cells**

Testes from adult CD1 mice (Charles River Italia) were used to prepare germ cells. After dissection of the albuginea membrane, testes were digested for 15 minutes in 0.25% (w/v) collagenase (type IX, Sigma) at room temperature with constant shaking. Digestion was followed by two washes in minimum essential medium (MEM, Gibco BRL), then seminiferous tubules were minced using a sterile blade and further
digested in MEM containing 1 mg/ml trypsin (Sigma) for 30 minutes at 30°C. Digestion was stopped by addition of 10% fetal calf serum and the released germ cells were collected after sedimentation (10 minutes at room temperature) of tissue debris. Germ cells were centrifuged for 10 minutes at 1000 g at 4°C and the pellet resuspended in 20 ml of elutriation medium [120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO3, 1.2 mM KH2PO4, 1.2 mM MgSO4 (7H2O), 1.3 mM CaCl2, 11 mM CO2. After 12 hours, cells were treated with either 0.5°C in a humified atmosphere containing 95% air and 5% CO2 for 10 minutes at 1000 g at room temperature) of tissue debris. Germ cells were centrifuged for 12 hours, washed with 10 ml of MEM, and resuspended in 50 μl of the fixative (4% paraformaldehyde). Cells were then incubated for 20 minutes at room temperature. Cells were then collected by centrifugation at 4°C and resuspended again in 50 μl of the fixative. After three washes, nuclei were blocked for 1 hour in PBS and slides were mounted in 50% glycerol in PBS.

**Cell culture and treatments**

After elutriation, pachytene spermatocytes were cultured in MEM, supplemented with 0.5% bovine serum albumin (BSA), 1 mM sodium pyruvate, 2 mM sodium lactate, in six-well dishes at a density of 106 cells/ml at 32°C in a humidified atmosphere containing 95% air and 5% CO2. After 12 hours, cells were treated with either 0.5 μM okadaic acid (OA) (Calbiochem) or equal volumes of the solvent DMSO, and culture was continued for up to 6 hours. In order to suppress the MAPK cascade, cells were preincubated for 12 hours prior to OA treatment with the specific inhibitor of MEK1/2 kinases U0126 (Calbiochem) at a concentration of 10 μM or with equal volumes of the solvent DMSO. For cytological and immunofluorescence analyses and kinase assays, aliquots of the same samples were taken and processed as described below.

**Plasmid construction**

cDNAs for N-terminal hemoagglutinin (HA)-tagged rat Rsk1, murine Rsk2 and human Rsk3 in pMT2 (Zago et al., 1996) were kindly provided by Christian Bjorbaek (Beth Israel Hospital, Boston, MA).
pGEX-3X-Nek21-272 and pGEX-3X-Nek2273-444, which carry the catalytic and regulatory domains of Nek2, respectively, were generated by RT-PCR of total RNA of spermatocytes and subsequent PCR of the full-length cDNA (GI 6754817), using appropriate primers. pGEX-3X-Nek21-272 contains the initial methionine of Nek2 and a new stop codon introduced at the end of the catalytic domain. pGEX-3X-Nek2273-444 contains a new methionine introduced upstream of the regulatory domain of Nek2 and the stop codon of Nek2. BamHI sites were introduced at both ends for subcloning into the BamHI site of pGEX-3X (Pharmacia).

**Glutathione S-Transferase (GST)-Nek2 regulatory domain fusion protein synthesis and purification**

Escherichia coli (BL21) transformed with pGEX-3X-Nek2 constructs were grown at 30°C in LB medium to an optical density (O.D. 600nm) of 0.5. Expression of recombinant proteins was induced by the addition of 0.5 mM isopropyl-1-thio-β-galactopyranoside for 4 hours at the same temperature. Cells were pelleted and lysed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 1 mM DTT, protease inhibitors, by probe sonication (three cycles of 1 minute each). Bacterial extracts were clarified by centrifugation at 12000 g and supernatant fractions were incubated with glutathione-Sepharose beads (Sigma, G 4510) for 1 hour at 4°C with constant shaking. After several washes in PBS, proteins were eluted with 50 mM Tris-HCl pH 8, containing 10 mM glutathione (Sigma, G 4251) and 1 mM DTT. Purified proteins were stored at −80°C in the same buffer containing 10% glycerol.

**RNA extraction and Northern blot analysis**

Total RNA was extracted from cells and tissues using the Trizol Reagent (Gibco BRL) and following the manufacturer’s instructions. Total RNA (20 μg) was extracted and separated on a 1.2% agarose/formaldehyde gel and blotted onto nylon membrane (Hybond-N, Amersham, UK) in 10x saline sodium citrate (SSC) buffer. The membrane was pre-hybridized at 42°C for 4 hours in a phosphate buffer solution (60 mM, pH 6.8) containing 50% formamide, 3x SSC, 10 mM EDTA (pH 7.2), 0.2% SDS and 5x Denhardt solution. Hybridization was carried out overnight under the same conditions with p90Rsk1, p90Rsk2 and p90Rsk3-cDNA clones radiolabeled with [32P]dATP by random primer labeling.

The membrane was washed once with 1x SSC, 0.1% SDS at room temperature and three times with 0.2x SSC, 0.1% SDS at 42°C before autoradiography.

**Western blot analysis**

Solubilized proteins were boiled for 5 minutes in SDS-PAGE sample buffer [62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (w/v) bromophenol blue], and resolved on 10% or 15% SDS-polyacrylamide gel electrophoresis after. Resolved proteins were transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were then saturated with 5% nonfat dry milk in PBS for 1 hour at room temperature and incubated with the primary antibody overnight at 4°C [mouse monoclonal anti-p-Erk (NEB, 1:500 dilution), rabbit anti-Erk1/2 (Santa Cruz, 1:1000 dilution), or goat polyclonal anti-p90Rsk1, anti-p90Rsk2 or anti-p90Rsk3 (Santa Cruz, 1:1000 dilution)]. Secondary antibody conjugated to horseradish peroxidase was incubated with the membranes for 1 hour at room temperature. Immunostained bands were detected by chemiluminescent method (Santa Cruz Biotech.).

**Chromatin staining**

Slides for chromatin analysis were prepared by modifying the procedures described by Meredith (Meredith, 1969). About 8x105 spermatocytes were lysated in 1 ml of 75 mM KCl solution and left 30 minutes at 37°C. About 0.5 ml of a fixative solution (one part acetic acid and three parts methanol) were added to the solution and incubated for 20 minutes at 4°C. Cells were then collected by centrifugation at 4000 g, resuspended in 0.5 ml fixative solution, incubated for 20 minutes at room temperature, collected by centrifugation at 4000 g and resuspended again in 50 μl of the fixative solution. Two or three drops of the cell suspension were squashed on a clean slide. DNA was stained with 5% Giemsa.

**Immunofluorescence analysis**

Slices of frozen adult testis were prepared using a microtome and placed on glass slides. Control or OA-treated spermatocytes were spotted on poly-L-lysine coated glass slides and fixed at room temperature for 15 minutes in 4% paraformaldehyde. Cells were permeabilized for 10 minutes in 0.1% TritonX-100 and blocked for 1 hour in PBS with 5% BSA. After three washes in PBS, cells were incubated over night at 4°C with mouse monoclonal anti-p-Erk (NEB, 1:100 dilution), or goat polyclonal anti-p90Rsk1, anti-p90Rsk2, or anti-p90Rsk3 (Santa Cruz, 1:400 dilution), as primary antibodies. After five washes in PBS, cells were incubated for 1 hour at 37°C with rhodamine-conjugate goat anti-mouse IgG (Calbiochem, catalog number 401217, 1:30 dilution) and rhodamine-conjugate donkey anti-goat IgG (Santa Cruz, catalog number sc-2094, 1:400 dilution). To stain DNA, Hoechst dye (Sigma) was added for the last 10 minutes at a final concentration of 0.1 mg/ml. Cells were washed extensively in PBS and slides were mounted in 50% glycerol in PBS.

**Immunofluorescence staining of meiotic nuclei spreads**

The procedures used for immunofluorescence analysis of meiotic prophase chromosomes were a slight modification of the technique reported by Dobson et al. (Dobson et al., 1994). In brief, cells were lysed in hypotonic salt (140 mM, pH 8.0), nuclei were attached to glass multwell slides and fixed in 2% paraformaldehyde for 6 minutes. After three washes, nuclei were blocked for 1 hour in PBS with 5% BSA. Fluorescence was performed as described above.
Immunoprecipitation experiments

Control or treated spermatocytes (approximately 2x10^6 cell/sample) were collected by centrifugation at 2000 g for 10 minutes, and washed twice in ice-cold PBS. Cells were homogenized in lysis buffer (25 mM Hepes, pH 7.5, 100 mM NaCl, 20 mM β-glycerophosphate, 15 mM EGTA, 15 mM MgCl_2, 0.1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin and 10 μg/ml aprotinin, 1 mM PMSF) and cytosolic fractions were collected after centrifugation at 15,000 g for 10 minutes at 4°C. For immunoprecipitation, 1 μg of goat polyclonal anti-p90Rsk1, anti-p90Rsk2, anti-p90Rsk3, anti-Nek2 antibodies or rabbit polyclonal anti-Erk1 (Santa Cruz Biotechnology) were preincubated for 60 minutes with a mixture of protein A- and protein G-Sepharose beads (Sigma) or only protein A with PBS containing 0.05% BSA, under constant shaking at 4°C. At the end of the incubation, the beads were washed twice with PBS and 0.05%BSA, twice with lysis buffer, and then incubated for 90 minutes at 4°C with the soluble spermatocyte cell-extracts (0.5 mg protein) under constant shaking. Sepharose beads-bound immunocomplexes were rinsed three times with PBS and eluted in SDS-sample buffer for western blot analysis, or washed twice with the appropriate kinase buffer for immunokinase assays (see below).

Immunokinase assays

Immunocomplexes bound to sepharose beads obtained from immunoprecipitation of cell extracts were eluted twice with either p90Rsks/Erk1 kinase buffer (50 mM Hepes, pH7.5, 5 mM β-glycerophosphate, 2 mM EGTA, 15 mM MgCl_2, 0.1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin and 10 μg/ml aprotinin) or Nek2 kinase buffer (20 mM Hepes, pH7.5, 5 mM β-glycerophosphate, 5 mM MnCl_2, 5 mM NaF, 0.1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Pellets were then incubated in the same kinase buffer with the addition of 10 μM 32Pγ-ATP (0.2 μCi/μl), 1 μg cAMP-dependent protein kinase inhibitor and the appropriate substrate (500 μM MBP-derived peptide (Santa Cruz, sc-3011) for Erk1; 100 μM S6 peptide (Calbiochem), 1 μg of histone H1 (SIGMA, Type III-S, H-5505) or 0.1 mg/ml Histone H3 (Boehringer) for p90Rsks; or 1 μg full-length MBP for Nek2. Reactions were carried on in a total volume of 50 μl for 20 minutes at 30°C, and were stopped either by adding SDS-sample buffer and boiling or by spotting 20 μl onto phosphocellulose paper (Whatman P-81) and immediately immersing it into 0.1% phosphoric acid. Paper squares were washed five times for 10 minutes each and air-dried. Radioactivity incorporated was determined by scintillation counting. Values were normalized for protein content, determined according to Bradford (Bradford, 1976). Samples diluted in SDS-sample buffer were separated on SDS-PAGE and the dried gel exposed to autoradiography.

RESULTS

Chromosome condensation requires MAPK activation

Incubation of mouse pachytene spermatocytes with OA induces a rapid condensation of chromatin and G2/M progression. We have previously shown that these two events can be separated: inhibition of MAPK pathway by the MEK1/2 inhibitor PD98005 suppresses chromatin condensation, whereas it does not completely suppress activation of MPF (Sette et al., 1999). We have used the specific inhibitor U0126 (Favata et al., 1998) to block activation of MAPK signaling in spermatocytes. This inhibitor is more specific than PD98059 and it can be used at one fifth the concentration (Fig. 1E). In control cells, only 4% of spermatocytes are in metaphase of the
After 6 hours of treatment with 0.5 μM OA approximately 40% of cells shows condensation of chromatin into metaphase chromosomes. The effect of OA is strongly inhibited by 12 hours pretreatment with 10 μM U0126 with only 11% of cells show metaphase chromosomes. Preincubation of spermatocytes with U0126 alone had no effect on the number of metaphases in the cell population. Approximately 300 cells were counted from representative microscope fields of each sample. Slides from three independent experiments were analyzed.

Table 1. OA-induced chromatin condensation during meiotic G2/M progression in mouse spermatocytes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spermatocytes in metaphase (%±s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.0±1.4</td>
</tr>
<tr>
<td>OA</td>
<td>40±3.2</td>
</tr>
<tr>
<td>U0126</td>
<td>3.0±2.3</td>
</tr>
<tr>
<td>OA+U0126</td>
<td>11±5.7</td>
</tr>
</tbody>
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Expression of p90Rsk1 and p90Rsk2 in mouse spermatogenesis

In Xenopus oocytes, the MAPK pathway is required for spindle formation, suppression of S phase, and to regulate the activity of APC and the intracellular levels of cyclin B. All these effects during oocyte maturation are mediated by the MAPK effector p90Rsk (Gross et al., 2000). In order to investigate the role of p90Rsk in male meiosis, we first studied the expression of the three mammalian p90Rsk isoforms (Frodin and Gammentiolft, 1999). Specific cDNA probes for northern blot analysis of p90Rsk1, p90Rsk2 and p90Rsk3 expression were obtained by PCR amplification as described in the Materials and Methods. Specificity of the probes was assessed by analysis of mRNA obtained from COS cells transfected with the three different isoforms (data not shown). p90Rsk1 transcript is absent in both pre-meiotic and post-meiotic spermatogenic cells (Fig. 2A). The mRNA transcript for p90Rsk2 was mainly detected in spermatogonia and in pachytene spermatocytes, whereas p90Rsk3 was mainly expressed in haploid round spermatids (Fig. 2A). To confirm the results obtained with mRNA analysis at the protein level, we performed immunoprecipitation experiments using antibodies specific to p90Rsk1, p90Rsk2 or p90Rsk3, and cell extracts from different spermatogenic cells. Immunoprecipitated proteins were analyzed by western blot with the same specific antibodies and we used cell extracts from COS cells transfected with p90Rsk1, p90Rsk2 or p90Rsk3 as positive controls. We were unable to detect p90Rsk1 in any of the germ cells analyzed, confirming that this isoform is not expressed in the testis (Fig. 2B). A specific band of 82 kDa that corresponds to p90Rsk2 was detected in cell extracts from spermatogonia and spermatocytes (Fig. 2B). A faint band was also detected in round spermatids, and it could either reflect the small amount of mRNA detected in these cells (Fig. 2A) or derive from contaminating spermatocytes and/or spermatogonia in the spermatid cell population. Confirming the mRNA analysis, p90Rsk3 was mainly expressed in round spermatids (Fig. 2B), and to a smaller extent in spermatogonia.

To validate the observations obtained using isolated germ cells and to avoid problems caused by small levels of cross contamination in the cell populations used, we investigated the expression of p90Rsk isoforms directly in situ by immunoprecipitation of soluble protein extracts (500 μg) obtained with mRNAs analysis at the protein level. Immunoprecipitation of soluble protein extracts (500 μg) obtained from same cell populations as in A was performed using antibodies specific to p90Rsk1, p90Rsk2 or p90Rsk3, and immunoprecipitated proteins were analyzed by western blot using the same specific antibodies. As positive control, immunoprecipitated proteins obtained from COS cells transfected with recombinant p90Rsk1, p90Rsk2 or p90Rsk3 were used as control for antibody specificity. Molecular weights were calculated using the Gibco Benchmark protein ladder as standard.
immunofluorescence analysis of testis histological sections. As shown in Fig. 3, p90Rsk2 was specifically expressed in spermatogonia and in spermatocytes, which are positioned, respectively, inside and just above the basal compartment of the tubule. As suggested by northern and western analyses, p90Rsk3 expression was restricted to mitotic spermatogonia and to haploid spermatids, but it was absent in meiotic spermatocytes. p90Rsk1 was absent in all testicular germ cells (Fig. 3).

**p90Rsk2 is activated during the OA-induced meiotic G2/M transition**

The stage-specific expression of p90Rsk2 during spermatogenesis suggests that it could play a role in meiotic cells. To study whether p90Rsk2 is activated during the OA-induced G2/M progression of mouse spermatocytes, the kinase was immunoprecipitated from extracts of either control or OA-treated cells, and the activity was assayed using S6 peptide and 32P-γ-ATP as substrates. We observed a 10-fold increase of p90Rsk2 activity in spermatocytes incubated with OA for 4-6 hours (Fig. 4). As a control for specificity of immunoprecipitation and p90Rsk2 activation, we also used antibodies directed against the other two p90Rsk isoforms. The anti-p90Rsk1 did not precipitate any S6 activity from cell extracts (Fig. 4), although it could immunoprecipitate recombinant p90Rsk1 from transfected COS cell extracts (Fig. 2B). The anti-p90Rsk3 antibody precipitated a small amount of S6 kinase activity from both control and OA-treated spermatocyte extracts (Fig. 4), which could be due to small amounts of p90Rsk3 derived from contaminating round spermatids in the spermatocyte cell population (see Fig. 1).

p90Rsk kinases can be activated by at least two pathways in several cell systems: the Erk1/2 pathway and the PDK1 pathway (Frodin and Gammentolft, 1999; Gross et al., 1999). We tested whether the Erk1/2 pathway plays a role in p90Rsk2 activation during OA-induced G2/M male meiotic progression. Preincubation of spermatocytes with the MEK1/2 inhibitor U0126 (10 μM) completely suppresses Erks activation by OA (Fig. 1E). Under this condition, activation of p90Rsk2 by OA-treatment was also completely suppressed, as measured by both S6 kinase activity after immunoprecipitation (Fig. 5A) and electrophoretic mobility in SDS-PAGE, where phosphorylated and activated p90Rsk2 shows an upward mobility shift (Fig. 5B). These results suggest that p90Rsk2 activation during G2/M progression of spermatocytes is entirely mediated by the MAPK pathway.

**Immunolocalization of p90Rsk2 in mouse spermatocytes**

We have previously shown that activation of the MAPK pathway is required for efficient chromosome condensation during the OA-induced G2/M progression and that Erk1

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**Fig. 3.** Differential expression of p90Rsk2 and p90Rsk3 in mouse spermatocytes and spermatids. Histological sections obtained from adult testis were stained with antibodies directed against the three p90Rsk isoforms. Immunostaining was carried out using the anti-p90Rsk1, anti-p90Rsk2 or anti-p90Rsk3 antibody and a rhodamine-conjugated secondary antibody as described in the Materials and Methods. Sections were also stained with Hoechst 3332 to detect chromatin (right panels). In all panels, L stands for lumen of the tubule and B for basal lamina. In the anti-p90Rsk2 panels, the arrowheads indicate a representative spermatocyte, whereas in the anti-p90Rsk3 panels, the arrowheads point to a representative elongated spermatid (central tubule) and a representative round spermatid (lower tubule). The layer of spermatogonia just above the basal lumina is stained by both the anti-p90Rsk2 and the anti-p90Rsk3 antibody.
Nek2 activation colocalizes with the meiotic spindle in close proximity with the condensed chromosomes (Sette et al., 1999). As p90Rsks are downstream effectors of Erks, it is possible that p90Rsk2 plays a direct role in chromatin condensation. As a first step, we investigated the subcellular localization of p90Rsk2 in mouse spermatocytes. In control cells, the distribution of p90Rsk2 was mainly nuclear (Fig. 6), and after OA treatment the staining was more punctuate than in control cells and it seemed to localize with the condensed chromosomes (Fig. 6). To investigate whether p90Rsk2 was indeed associated with meiotic chromosomes, cells were lysed in hypotonic solution to disrupt cytoplasmic and nuclear membranes, and chromatin was dispersed directly on a microscope slide for immunofluorescence analysis. Metaphase chromosomes were strongly stained by the p90Rsk2 antibody, demonstrating a tight association of the kinase with condensed chromatin (Fig. 7A). Staining with the anti-p90Rsk2 antibody was specific, because neither the anti-p90Rsk1, which is absent in these cells, nor secondary antibody alone stained metaphase chromosomes (data not shown).

Since p90Rsk2 is activated by Erks (see Fig. 5), we tested whether phosphorylated and activated Erks were also associated with meiotic chromosomes. To this end, immunofluorescence analysis of dispersed chromatins was carried out using an antibody specific for the phosphorylated-activated forms of Erk1/2. We found that, similar to p90Rsk2, active Erks also colocalized with metaphase chromosomes (Fig. 7B), suggesting that an active Erk-p90Rsk complex forms on meiotic chromatin and plays a role during condensation into metaphase chromosomes.

Histone H3 phosphorylation is not mediated by the MAPK pathway in mouse spermatocytes

During the G2/M progression of mitotic and meiotic cells, histone H3 is phosphorylated at Ser10 (Wei et al., 2000; Cobb et al., 1999b), and phosphorylation is thought to mediate interaction of nucleosomes with factors that regulate both gene transcription and chromatin condensation (reviewed by Cheung et al., 2000). It has been shown that p90Rsk2 is able to phosphorylate H3 at Ser10 in vitro, and that p90Rsk2-mediated phosphorylation of H3 is necessary for gene transcription in mitotic cells (Sassone-Corsi et al., 1999).

To study the role of the MAPK pathway in H3 phosphorylation during male meiosis, we used two approaches. First, p90Rsk2 was immunoprecipitated from control or OA-treated spermatocytes and purified H3 was used as substrate in an in vitro immunokinase assay. Phosphorylated H3 was then detected by western blot analysis using an antibody raised against phosphorylated Ser10 of H3. As shown in Fig. 8A, p90Rsk2 was able to phosphorylate H3 in vitro, and phosphorylation was induced by activation of the enzyme after treatment of cells with OA. Inhibition of the MAPK pathway by U0126, which prevents activation of p90Rsk2 by OA, blocks phosphorylation of H3 in this in vitro kinase assay.
demonstrating that the activity is due to p90Rsk2 (Fig. 8A). Second, we studied the impact of activation of MAPKs on H3 phosphorylation in vivo by western blot analysis of extracts of cells after different treatments. In control spermatocytes, H3 is not phosphorylated at Ser10, but H3 phosphorylation was strongly induced after 6 hours treatment with OA (Fig. 8B). However, preincubation of spermatocytes with the MAPK inhibitor U0126 did not prevent OA-induced H3 phosphorylation, indicating that Erks and p90Rsk2 are not required for this event in vivo (Fig. 8B). As activation of Erks and p90Rsk2 are necessary for efficient chromatin condensation in these cells, these data suggest that H3 phosphorylation correlates with appearance of metaphase chromosome but it is not sufficient to trigger this event in male meiotic cells.

**Activation of Nek2 in mouse spermatocytes**

The Ser/Thr protein kinase NIMA was first isolated in *Aspergillus nidulans* and shown to play a role in chromosome condensation during mitosis (Fry et al., 1995). The NIMA murine homolog Nek2 is expressed in pachytene and diplotene spermatocytes where it is found associated with meiotic chromosomes (Tanaka et al., 1997; Rhee and Wolgemuth, 1997). During the G2/M progression triggered by OA, Nek2 is activated with a time course similar to that of MPF and Erk1 (Rhee and Wolgemuth, 1997); however, the roles played by Nek2 and the pathway leading to its activation in spermatocytes are unknown.

To test whether the MAPK pathway is required for Nek2 activation, spermatocytes were preincubated in the presence or absence of 10 μM U0126 for 12 hours and then treated for 6 hours with or without 0.5 μM OA. Nek2 activity was assayed using either MBP or casein as substrate. Activation of Nek2 by OA correlated with chromosome condensation and G2/M progression as previously reported (Rhee and Wolgemuth, 1997) (Fig. 9A). Interestingly, inhibition of the MAPK pathway by preincubation with U0126 completely blocked OA-induced activation of Nek2, whereas the basal activity of Nek2 was not affected in control cells (Fig. 9A). This result suggests that activation of ERKs and p90Rsk2 is required for activation of Nek2 during G2/M progression of mouse spermatocytes.

To investigate whether p90Rsk2 was able to induce activation of Nek2, we used an in vitro approach. As a source of active and inactive p90Rsk2, COS cells were transfected with a p90Rsk2 cDNA and incubated respectively with or without OA for 3 hours to induce activation of p90Rsk2 (~10-fold, data not shown). Immunopurified active or inactive p90Rsk2 was then incubated for 30 minutes at 30°C with cytosolic extracts from control spermatocytes. After the incubation, Nek2 was immunoprecipitated from the cytosolic extracts and its activity tested using MBP as substrate. We found that Nek2 was activated after incubation with active p90Rsk2, when compared with Nek2 incubated with the inactive form, indicating that p90Rsk2 is able to stimulate the activity of Nek2 in vitro (Fig. 9B). Similar results were also obtained using casein as substrate for Nek2 activity (data not shown). When cytosolic extracts were immunodepleted of Nek2 before incubation with p90Rsk2, no MBP activity could be immunoprecipitated with anti-Nek2 antibodies, demonstrating that the MBP kinase activity was not due to nonspecific binding of a kinase to the beads (data not shown).

Finally, we tested whether p90Rsk2 was able to phosphorylate purified Nek2 in vitro. We were unable to express and purify full length GST-Nek2 from *E. coli*. Therefore, we produced two fusion proteins, one containing the N-terminal kinase domain of Nek2 (amino acids 1-272) and another containing the C-terminal domain (amino acids 273-444) (Fig. 9C), which is supposed to play a regulatory role (Rhee and Wogelmuth, 1997). p90Rsk2 was immunopurified using a specific antibody and in vitro kinase assays were performed using GST-Nek21-272 or GST-Nek2273-444 as substrates in the presence of 32P-g-ATP. We found that GST-Nek21-272 was phosphorylated by activated p90Rsk2 (Fig. 9D), whereas GST-Nek2273-444 (Fig. 9D) or GST alone (data not shown) was not. The highly phosphorylated band at 30-35 kDa (indicated by an arrowhead in Fig. 9D) is due to

**Fig. 6.** Subcellular localization of p90Rsk2 in mouse spermatocytes. The panels on the left show the immunofluorescence analysis of control (top) or OA-treated (6 hours with 0.5 μM, bottom) cells. Immunostaining was carried out using the anti-p90Rsk2 antibody and a rhodamine-conjugated secondary antibody as described in the Materials and Methods. Cells were also stained with Hoechst 33332 to detect chromatin (right panels).
phosphorylation of proteolytic fragments of GST-Nek2-272 that were routinely purified from _E. coli_ together with the full-length protein (arrowheads in Fig. 9C). As these fragments are much better substrates for p90Rsk2 in vitro than is the entire kinase domain (Fig. 9D), it is possible that full-length Nek2 may need a particular conformation to be phosphorylated by p90Rsk2.

The data presented demonstrate that activation of the MAPK pathway during the G2/M progression of mouse spermatocytes leads to phosphorylation and activation of Nek2 by p90Rsk2 (Fig. 10).

**DISCUSSION**

At the end of the pachytene stage of meiotic prophase, the synaptonemal complex dissolves and chromatin condenses into metaphase chromosomes, which align on the equatorial plate of the spindle. We have previously demonstrated that activation of the MAPK Erk1 is required for efficient chromatin condensation during the G2/M progression triggered by OA in mouse pachytene spermatocytes (Sette et al., 1999). We have extended these studies and identified p90Rsk2 as a downstream effector of the MAPK pathway in these cells. Furthermore, we show that Nek2, a serine-threonine kinase involved in chromatin condensation and centrosome duplication, is activated by the MAPK pathway and is a substrate of p90Rsk2 in vitro.

Three p90Rsk isoforms, p90Rsk1, p90Rsk2 and p90Rsk3, are expressed in mouse tissues (Frodin and Gammeltoft, 1999). Our northern blot, western blot and immunofluorescence analyses demonstrate that these isoforms are specifically expressed during spermatogenesis. p90Rsk2 is expressed during the mitotic and meiotic stages of spermatogenesis, from spermatogonia to pachytene spermatocytes, and its expression decreases or ceases in post-meiotic spermatids. p90Rsk3 is present at low levels in spermatogonia, is absent (or expressed at very low levels) in meiotic spermatocytes and is abundant in post-meiotic cells, suggesting that it could play a role during differentiation of round spermatids into mature sperm. p90Rsk1 is not expressed in germ cells at any stage examined.

In mitotic cells and in maturing _Xenopus_ oocytes, p90Rsk2 was found to be the main effector of Erks. Indeed, activation of p90Rsk2 is required for all the known effects exerted by the MAPK pathway in meiotic oocytes, such as formation of a meiotic spindle during the first division, suppression of DNA replication between the two metaphases, and inhibition of the cycle at metaphase of the second division after progesterone-induced maturation (Bhatt and Ferrell, 1999; Gross et al., 1999; Gross et al., 2000). We found that p90Rsk2 was also activated by Erks during mouse male meiosis. However, our data suggest that in spermatocytes, activation of the MAPK pathway is also important for triggering chromosome condensation, and not only for the maintenance of chromatin condensed between the two meiotic divisions and for the prevention of DNA synthesis during interphase. The role of MAPK at this earlier meiotic stage could represent another level of the dimorphism observed between male and female meiosis in higher eukaryotes (Handel and Eppig, 1998).

In agreement with the role of Erk1 activation during Fig. 7. Activated Erk1/2 and p90Rsk2 associate with meiotic chromosomes. Chromatin and/or chromosomes were obtained by hypotonic treatment of spermatocytes and were attached directly on a microscope slide for immunofluorescence analysis as described in the legend to Fig. 5. (A) Chromatin was stained with anti-p90Rsk2 antibody (right panels) or Hoechst dye (left panels). (B) Chromatin was stained with anti-phosphoErk1/2 antibody (right panels) or Hoechst (left panels). Both p90Rsk2 and activated Erks colocalize with meiotic chromosomes.
chromatin condensation (Sette et al., 1999), we show that activated Erks localize in close association with condensed chromosomes at metaphase in mouse spermatocyte. Furthermore, their effector p90Rsk2 is always nuclear, and it also associates with condensed chromosomes at metaphase. Therefore, both Erks and p90Rsk2 are able to directly or indirectly interact with chromatin and regulate its state of condensation, probably by phosphorylating proteins that modulate nucleosomal assembly (Fig. 10). Whereas interaction of activated Erks with chromosomes had been previously reported in mitotic cells (Zecevic et al., 1998), where they associate with the kinetochores and the motor protein CENP-E, a similar localization for p90Rsk2 has never been observed and it could suggest the direction for searching new p90Rsk substrates. Recently, it has been shown that in *Xenopus* eggs p90Rsk phosphorylates and activates Bub1, an upstream

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**Fig. 8.** Histone H3 phosphorylation is not mediated by the MAPK pathway in mouse spermatocytes. (A) Immunokinase assay of p90Rsk2 activity using H3 as substrate. p90Rsk2 was immunoprecipitated as described in the legend to Fig. 4 from cells treated as indicated in the figure and in the text, and activity was assayed using 0.1 mg/ml H3 and 10 mM ATP as substrates. At the end of the incubation, proteins were separated on a 10% SDS-PAGE and analyzed by western blot using either anti-phospho-H3 to detect phosphorylation of Ser10 in H3, or anti-p90Rsk2 to verify that equal amounts of enzyme were used in the assay. (B) Western blot analysis of extracts (20 μg) of cells treated as indicated in the figure and in the text using either the anti-phosphoH3 antibody to detect phosphorylation of Ser10 in H3 in intact cells, or the anti-H3 antibody to quantify the amount of H3 in the samples.

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**Fig. 9.** Activation of the Ser/Thr protein kinase Nek2 in mouse spermatocytes is MAPK dependent. (A) Immunokinase assay of Nek2 activity. Nek2 was immunoprecipitated from control or OA-treated cells preincubated or not with U0126 (10 μM for 12 hours) and incubated for 20 minutes at 30°C in the presence of 1 μg full-length MBP and 10 μM [γ-32P]-ATP as substrates. Reactions were terminated by adding SDS-sample buffer, samples were boiled for 5 minutes and protein were separated on a 13% SDS-PAGE. The dried gel was then autoradiographed. (B) Reconstitution of Nek2 activation in vitro. Active and inactive p90Rsk2 were immunopurified as described in the Materials and Methods, and incubated with cytosolic extracts from control spermatocytes for 30 minutes at 30°C in the presence of 10 μM ATP. At the end of the incubation, cytosolic extracts were separated by centrifugation and Nek2 was immunopurified using 1 mg anti-Nek2 antibody and proteinA-sepharose beads. The activity of Nek2 was assayed as described in A and the dried gel was autoradiographed. (C) Coomassie Blue staining of GST-Nek21-272 and GST-Nek2273-444 purified from *E. coli* that were used in D as substrates for p90Rsk2. Arrowheads on the left side point to degradation products routinely observed in purified GST-Nek21-272. (D) In vitro assay for phosphorylation of GST-Nek21-272 and GST-Nek2273-444 by p90Rsk2. Active or inactive immunopurified p90Rsk2 was incubated with GST-Nek2 proteins for 30 minutes at 30°C in the presence of 10 μM ATP. The reaction was terminated adding SDS-PAGE sample buffer and proteins were separated on a 10% SDS-PAGE. The gel was dried and autoradiographed. Arrowhead on the left shows the position of GST-Nek21-272 degradation products.
component of the kinetochore attachment checkpoint, suggesting that APC inhibition and the cytostatic activity exerted by the MAPK pathway might be mediated by this checkpoint protein (Schwab et al., 2001). Our data suggest that in mammalian cells, in addition to regulation of the exit from metaphase after the attachment of the kinetochores to the spindle, the MAPK pathway also regulates earlier steps involved in assembly of chromatin into metaphase chromosomes. A candidate for regulation of chromatin assembly is histone 3 (H3). It has been reported that phosphorylation of H3 at Ser10 temporally correlates with entry into metaphase and chromatin condensation during both mitosis and meiosis (Hsu et al., 2000; Chadee et al., 1999; Cobb et al., 1999b). Several protein kinases, including MAPKs, p90Rskks, Aurora A and B kinases have been shown to phosphorylate H3 in vitro and/or in vivo (Sassone-Corsi et al., 1999; Murnion et al., 2001; Scrittori et al., 2001). However, the role played by p90Rsk in H3 phosphorylation is not completely understood. Sassone-Corsi et al. (Sassone-Corsi et al., 1999) have reported that p90Rsk2 phosphorylates H3 in mitotic cells and that this phosphorylation is required for induction of early genes in G1. However, p90Rsk2 does not seem to be required for phosphorylation of H3 later in the cell cycle, as inhibition of the MAPK pathway does not block this event during the G2/M progression in Xenopus egg cycling extracts (Murnion et al., 2001). Our data extend these results to mammalian meiotic cells, because we have demonstrated that inhibition of MAPKs and p90Rsk2 activation does not affect phosphorylation of H3 during meiotic G2/M transition. Because inhibition of the MAPK pathway strongly affects chromatin condensation, our data also indicate that H3 phosphorylation is not sufficient to trigger chromosome assembly. Indeed, a similar observation has also been reported by Murnion et al. (Murnion et al., 2001) in mitotic cycling extracts, where stimulation of H3 phosphorylation in interphase cytosols was not sufficient to drive chromosome condensation or targeting of condensins to chromatin. Therefore, proteins other than H3 must be targets of p90Rsk2 in vivo, and play a role in proper condensation of chromatin during both mitosis and meiosis.

In this study, we have identified Nek2 as one of the possible effectors of p90Rsk2 during meiotic chromosome condensation. Nek2 is a member of the NIMA kinases, for which a role in chromosome condensation and centrosome duplication has been suggested in organisms as diverse as yeast and vertebrates (Fry and Nigg, 1995; Fry et al., 1998a; Fry et al., 1998b; Uto and Sagata, 2000). In Aspergillus nidulans, mutations in NIMA arrest cells in late G2 (Morris, 1976), even when Cdc2 is activated, indicating that NIMA is part of a Cdc2-independent mitotic pathway (Osmani et al., 1991). However, although not required for its basal activity, CyclinB-Cdc2 phosphorylates NIMA and stimulates its kinase activity, suggesting that a positive crosstalk between the two pathways exists and could play a role in coordinating mitotic events (Ye et al., 1995). In metazoan cells, the expression and activity of the NIMA homologues (Nek1, Nek2 and Nek3) are cell cycle regulated during mitosis (Fry and Nigg, 1995; Fry and Nigg, 1997). Nek2 expression levels and kinase activity are high in late S-phase and G2, decrease at metaphase, and are absent in G1 (Fry et al., 1995). Furthermore, inappropriate activation of Nek2 triggers premature condensation of chromatin in a Cdc2-independent fashion (Lu and Hunter, 1995; O’Connell et al., 1994), suggesting that its physiological role is to control the timing of chromatin condensation at the end of S-phase. It was previously observed that Nek2 is specifically expressed in mouse pachytene spermatocytes, and that its activity increases during the G2/M progression triggered by OA in these cells (Tanaka et al., 1997; Rhee and Wogelmuth, 1997). We demonstrate that Nek2 activity is modulated by the MAPK pathway in vivo, and that it can be stimulated by p90Rsk2 in an in vitro reconstitution experiment. Furthermore, we show that a recombinant GST-Nek2 protein, which contains the N-terminal half of the kinase (including the kinase domain), is a substrate for p90Rsk2 in vitro. These results indicate a connection between the MAPK pathway and the pathway that leads to chromosome condensation at the end of the first meiotic prophase (Fig. 10).

Little is known about upstream regulators of Nek2 or pathways that lead to its activation during meiosis or mitosis. Therefore, activation of Nek2 by Erks-p90Rsk2 represents the first connection between a NIMA kinase and pathways regulated by extracellular signals. Although we have used OA to trigger MAPK activation and meiotic progression, several observations suggest that the events described here have a physiological relevance. First, chromosomes condense as normal meiotic bivalents (Wiltshire et al., 1995), showing that this progression occurs following the physiological route, and inhibition of the MAPK pathway by U0126 interferes with this event. Therefore, we hypothesize that MAPK activation is not a mere epiphenomenon caused by treatment of cells with a serine-threonine phosphatase inhibitor, but it may also play a role during the natural meiotic progression. Second, MAPK is necessary for the activation of Nek2, a protein known physiologically to induce chromatin condensation and centrosome duplication. Third, we were able to isolate

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**Fig. 10.** Hypothetical model of the activation of Nek2 and induction of chromatin condensation by Erk1 and p90Rsk2. Erk1 may be activated by local extracellular signals when spermatocytes are mature to enter the meiotic divisions. Activation of Erk1 allows its translocation into the nucleus where it phosphorylates and activates p90Rsk2. Activated Erk1 and p90Rsk2 associates with chromatin, and p90Rsk2 phosphorylates and activates the chromatin-bound Nek2. Activation of Nek2 triggers chromatin condensation into metaphase chromosomes.
spermatocytes at late pachytenne or diplotenone stage and have observed an increase in MAPK activity in these cells (S. D., P. R., R. G. and C. S., unpublished), reinforcing the idea that this pathway plays a role in male meiosis.

It is not currently known what stimuli physiologically lead to MAPK activation during the prophase of male meiosis; however, it is conceivable to hypothesize the involvement of paracrine factors temporally expressed in the semiferous tubule. Indeed, although meiotic events such as crossing over and DNA repair after recombination have already occurred at mid-pachytenne (Wiltshtire et al., 1995; Cobb et al., 1999a), spermatocytes wait for several days at this cell-cycle stage before entering metaphase. This time is used to accumulate RNAs and proteins that will allow two subsequent cell divisions without intervening growth. Further meiotic progression might depend on appropriate signals from the neighboring germ cells or the nursing Sertoli cell when the spermatocyte has reached its mature size. The observation that activation of extracellular-regulated enzymes such as Erk1 and p90Rsk2 is required for activation of Ne2k and proper chromosome condensation in pachytenne spermatocytes supports such hypothesis. Future studies will be aimed at identifying physiological factors involved in regulation of the MAPK pathway and meiotic progression in mouse spermatocytes.

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