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Phosphorylation of High-Mobility Group Protein A2 by Nek2 Kinase during the First Meiotic Division in Mouse Spermatocytes

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The mitogen-activated protein kinase (MAPK) pathway is required for maintaining the chromatin condensed during the two meiotic divisions and to avoid a second round of DNA duplication. However, molecular targets of the MAPK pathway on chromatin have not yet been identified. Here, we show that the architectural chromatin protein HMGA2 is highly expressed in male meiotic cells. Furthermore, Nek2, a serine-threonine kinase activated by the MAPK pathway in mouse pachytene spermatocytes, directly interacts with HMGA2 in vitro and in mouse spermatocytes. The interaction does not depend on the activity of Nek2 and seems constitutive. On progression from pachytene to metaphase, Nek2 is activated and HMGA2 is phosphorylated in an MAPK-dependent manner. We also show that Nek2 phosphorylates in vitro HMGA2 and that this phosphorylation decreases the affinity of HMGA2 for DNA and might favor its release from the chromatin. Indeed, we find that most HMGA2 associates with chromatin in mouse pachytene spermatocytes, whereas it is excluded from the chromatin upon the G2/M progression. Because *hmga2−/−* mice are sterile and show a dramatic impairment of spermatogenesis, it is possible that the functional interaction between HMGA2 and Nek2 plays a crucial role in the correct process of chromatin condensation in meiosis.

INTRODUCTION

The endpoint of meiosis is the production of four haploid cells from a single diploid precursor. An important feature of the meiotic cell cycle is the maintenance of condensed chromatin during the short interphase that separates metaphase I from metaphase II, which is required to prevent DNA duplication, and it allows the reduction of the genome from a diploid to a haploid state (Roeder, 1997; Handel and Eppig, 1998; Petronczki *et al*., 2003). In amphibian oocytes, the activation of the mitogen-activated protein kinase (MAPK) pathway by Mos, a meiotic-specific MAPK kinase kinase, is required for chromatin condensation during the two meiotic divisions: indeed, inhibition of MAPK activity by the mitogen-activated protein kinase kinase 1/2 inhibitor U0126 elicits decondensation of chromatin after anaphase I and the onset of DNA duplication in *Xenopus* oocytes (Gross *et al*., 2000; Tunquist and Maller, 2003). In these studies it was also shown that p90Rsk2 is the effector of the Mos-MAPK cascade and mediates the suppression of interphase, the assembly of the meiotic spindle and allows cyclin B accumulation (Gross *et al*., 2000).

The role played by the MAPK pathway in mammalian meiosis is less clear. Deletion of the *mos* gene has no apparent effect in male meiosis in the mouse, whereas it alters the meiotic divisions of oocytes (Colledge *et al*., 1994; Hashimoto *et al*., 1994). It was shown that *mos−/−* oocytes progress normally through the first meiotic division and extrude the first polar body, but they fail to arrest at metaphase II, a prerequisite for physiological fertilization by the sperm. Thus, it was proposed that in mammalian meiosis the MAPK pathway plays a role only in establishing the cytostatic activity that maintains the oocyte arrested at metaphase II until fertilization occurs (Sagata, 1997; Tunquist and Maller, 2003). However, a closer look at the phenotype of *mos−/−* oocytes revealed that meiotic failure was more complicated than initially proposed. Choi and colleagues observed that the first meiotic division of these oocytes often resembles a mitotic division, with production of an abnormally large polar body that often undergoes further cleavage (Choi *et al*., 1996). Activation of the MAPK pathway in meiosis is also important for the asymmetric position of the spindle, which ensures unequal segregation of cytoplasm between the oocyte and the polar body (Verlhac *et al*., 1996, 2000). Hence, besides chromatin condensation and DNA duplication, spindle assembly is also under the control of the MAPK pathway in mammalian meiosis.

The lack of in vitro culture systems to study male meiotic progression has hampered the study of the molecular mechanisms involved in the mammalian spermatocyte cell cycle (Handel and Eppig, 1998). However, hints on the regulation of the male meiotic cell cycle started to occur when it was demonstrated that pachytene spermatocytes can be induced to enter metaphase in culture by treatment with the serine-threonine phosphatase inhibitor okadaic acid (OA) (Wilt-

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shire *et al*., 1995; Cobb *et al*., 1999). Metaphase chromosomes obtained by this treatment are normal bivalents in which crossing over is completed, the synaptonemal complex has dissolved, and chiasmata are present (Wiltshire *et al*., 1995). Spermatocyte G2/M progression is accompanied by activation of maturation promoting factor (MPF), the cyclinB–cdc2 complex (Wiltshire *et al*., 1995), and MAPKs (Sette *et al*., 1999). In particular, activation of the MAPK pathway is required to ensure efficient chromatin condensation into metaphase chromosomes (Sette *et al*., 1999). In this regard, it was shown that a chromatin-associated protein, the NIMA-like kinase Nek2, was also activated during the G2/M progression of mouse spermatocytes (Rhee and Wolgemuth, 1997).

NIMA kinase is required, in addition to Cdc2, for the G2/M progression and chromatin condensation during mitosis in *Aspergillus nidulans* (Osmani *et al*., 1991). We have observed that the MAPK pathway triggers the sequential activation of p90Rsk2 and Nek2 in mouse spermatocytes, thus suggesting that a kinase cascade involving Nek2 plays a role in chromatin condensation in male meiosis (Di Agostino *et al*., 2002). However, it is unclear whether Nek2 directly participates to chromatin condensation by phosphorylating proteins associated to DNA. Recently, mouse animal models have indicated that DNA architectural proteins belonging to the HMG superfamily are required for spermatogenesis. It was found that deletion of *hmga2* and *hmgb2*, which belong to two different HMG families (Bustin, 1999), produce mice that are either sterile or subfertile and display spermatogenic defects and low sperm production (Ronfani *et al*., 2001; Chieffi *et al*., 2002). HMGA and B proteins act as architectural factors in the chromatin, producing bends that expose DNA to transcriptional factors. Furthermore, their modification by phosphorylation and/or acetylation causes transcriptional regulation and a change in their affinity for DNA (Bustin, 1999). Interestingly, although HMGA1 and HMGA2 are highly expressed in the actively dividing embryonic tissues, their expression in the adult is confined to the testis and few other tissues (Fedele *et al*., 2001); however, their specific function in mouse spermatogenesis remains unknown.

In this work, we demonstrate a specific interaction between Nek2 and HMGA2 in mouse spermatocytes: the interaction is direct and requires the C-terminal regulatory region of the kinase. HMGA2 is phosphorylated in vivo in a MAPK pathway-dependent manner during the G2/M progression of mouse spermatocytes. Moreover, we show that Nek2 directly phosphorylates HMGA2 in vitro and that phosphorylation decreases the affinity of HMGA2 for DNA. Because HMG proteins are abundant components of chromatin that modulate DNA conformation and gene expression (Bustin, 1999), our data suggest that posttranslation modification of HMGA2 by the MAPK pathway can be part of the mechanisms leading to chromosome condensation in male meiosis.

MATERIALS AND METHODS

Cell Isolation, Culture, and Treatments

Testes from 18–20-d-old CD1 mice (Charles River Italica, Calco, Italy) were used to obtain pachytene spermatocytes by elutriation technique as described previously (Sette *et al*., 1999). Spermatogonia were obtained from mice 8–9 d old and round spermatids from 30 d old testes as described previously (Rossi *et al*., 1993). After elutriation, pachytene spermatocytes were cultured in minimal essential medium, supplemented with 0.5% bovine serum albumin (BSA), 1 mM sodium pyruvate, 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 12 h, cells were treated with 0.5 μM OA (Calbiochem, San Diego, CA) or equal volumes of the solvent dimethyl sulfoxide (DMSO), and culture was continued for an additional 6 h to induce metaphase I entry (Wiltshire *et al*., 1995). At the end of the incubation, cells were collected by centrifugation and immediately frozen at −80°C.

Plasmids Construction

pGEX-3X–Nek2$_{273-444}$ encoding the regulatory domain of Nek2 fused to glutathione *S*-transferase (GST) has been described previously (Di Agostino *et al*., 2002). pGEX-4T1–Nek2$_{272-294}$, pGEX-4T1–Nek2$_{272-369}$, and pGEX-4T1–Nek2$_{294-444}$ were obtained by polymerase chain reaction and cloned into the *Bam*HI and *Eco*RI sites of pGEX-3X as described previously (Di Agostino *et al*., 2002). Full-length Nek2 cDNA was obtained by reverse transcription-polymerase chain reaction from spermatocyte total RNA (GI 6754817) and cloned in the *Bam*HI site of the pCMV5 eukaryotic expression vector. The myc-epitope was inserted at the N terminus by cloning Nek2 cDNA in the *Bam*HI site of pCDNA3-myc. A catalytically inactive mutant of Nek2 (Nek2$_{K37R}$) (Fry *et al*., 1995) was created by site-directed mutagenesis of pCDNA3N2myc-Nek2. Mutagenic oligonucleotides were as follows: forward, 5′AGATATT-AGTTTGGAGAGAACTTGACTATGGC3′; and reverse, with the underlined codon corresponding to residue 37 in wild-type Nek2. The sequence of wild-type and mutant Nek2 were confirmed by sequence analysis. The pET2c/A2 fusion expression plasmid for expression of His-HMGA2 and pECEFL-HA-HMGA2 for expression in eukaryotic cells have been described previously (Baldassarre *et al*., 2001)

Bacterial Protein Expression and Purification

Escherichia coli cells (BL21-DE3) were transformed with the appropriate plasmid, grown at 37°C in LB medium to an optical density (600 nm) of 0.4, and induced with 0.5 mM isopropyl-1-thio-β-galactopyranoside for 3 h at the same temperature. Cells were harvested by centrifugation and lysed in ice-cold phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 1 mM dithiothreitol (DTT), protease inhibitors, by probe sonication (3 cycles of 1 min). After centrifugation at 12,000 × *g*, supernatant fractions were incubated with either glutathione-Sepharose beads (G 4510; Sigma-Aldrich, St. Louis, MO), for GST-Nek2, or Nichel-Agarose beads (QIAGEN, Valencia, CA), for His-HMGA2, for 1 h at 4°C under constant shaking. After washes in PBS, GST-fusions were eluted with either 100 mM Tris-HCl, pH 8, 250 mM NaCl containing 10 mM glutathione (G 4251; Sigma-Aldrich), whereas His-HMGA2 was eluted with 500 mM imidazole and dialyzed against PBS overnight. Purified protein was stored at −80°C in the same buffer also containing 10% glycerol.

Transfection of Human Embryonic Kidney (HEK)293 Cells and Preparation of Cells Extracts

HEK293 were cultured in 10-cm-diameter dishes and transfected with the calcium phosphate method. Transfected HEK293 were incubated with or without 0.5 μM OA for the last 2 h to activate Nek2. Thirty hours after transfection, cells were collected in lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM KCl, 5 mM $MnCl_2$, 5 mM EGTA, 2 mM EDTA, 20 mM β-glycerophosphate 0.1 mM sodium orthovanadate, 1 mM DTT, 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 0.1% Nonidet P-40, 30 μg/ml DNase) and homogenized. After 30 min on ice, cell lysates were centrifuges for 10 min at 10,000 × *g*, and the supernatants were used for further studies. The same protocol of cell lysis was used for germ cell lysates.

Western Blot Analysis

Solubilized proteins were boiled for 5 min in SDS-PAGE sample buffer [62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (wt/vol) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (wt/vol) bromphenol blue], and resolved on 10 or 12% SDS-PAGE. Western blot analysis was performed as described previously (Di Agostino *et al*., 2002). Primary antibodies, 2 h at room temperature, were as follows: mouse monoclonal anti-myc (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-His (1:1,000 dilution; QIAGEN); mouse monoclonal anti-hemagglutinin (HA) (1:1000 dilution; QIAGEN); rabbit anti-HMGA2 (25) (1:1,000 dilution) Baldassare, *et al*., 2001; rabbit anti-Nek2 R40 (provided by Dr. A.M. Fry, University of Leicester, Leicester, United Kingdom; 1:1,000 dilution); rabbit anti-extracellular signal-regulated kinase (Erk)1 (1:1,000 dilution; Santa Cruz Biotechnology); and goat anti-Nek2 (1:1,000 dilution; Santa Cruz Biotechnology). Secondary antibody conjugated to horseradish peroxidase 1:10,000; Santa Cruz Biotechnology) was incubated with the membranes for 1 h at room temperature. Immunostained bands were detected by chemiluminescent method (Santa Cruz Biotechnology.).

Immunofluorescence Analysis

Slices of frozen adult testis were prepared using a microtome and placed on glass slides and fixed at room temperature for 15 min in 4% paraformaldehyde. Samples were permeabilized for 10 min in 0.1% Triton X-100 and blocked for 1 h in PBS with 5% BSA. After three washes in PBS, samples were incubated over night at 4°C with rabbit anti-HMGA2 (25) (1:100 dilution) or

anti-Nek2 (R40; 1:100 dilution) as primary antibody. After five washes in PBS, cells were incubated for 1h at 37°C with rhodamine-conjugate goat anti-rabbit IgG (1:300 dilution; Calbiochem). To stain DNA, Hoechst dye (Sigma-Aldrich) was added for the last 10 min at a final concentration of 0.1 mg/ml. Samples were washed extensively in PBS and slides were mounted in 50% glycerol in PBS.

Immunoprecipitation Experiments

Control or treated spermatocytes (~2×10^6 cell/sample) were collected by centrifugation at $1000 \times g$ for 10 min, and washed twice in ice-cold 1× PBS. Cells were homogenized in Nek2 lysis buffer, and cytosolic fractions were collected as described above. For immunoprecipitation, 1 μg of mouse anti-myc, or rabbit polyclonal anti-Nek2 R40, or anti-HMGA2 antibodies were preincubated for 60 min with a mixture of protein A/G-Sepharose beads (Sigma-Aldrich) in PBS containing 0.05% BSA under constant shaking at 4°C. At the end of the incubation, the beads were washed twice with PBS/0.05% BSA, twice with lysis buffer, and then incubated for 90 min at 4°C with the soluble spermatocyte or HEK293 cell-extracts (0.5 mg of protein) under constant shaking. Sepharose bead-bound immunocomplexes were rinsed three times with lysis buffer and eluted in SDS-sample buffer for Western blot analysis, or washed twice with the appropriate kinase buffer for immunokinase assays.

Immunokinase Assays

Immunocomplexes bound to Sepharose beads obtained from immunoprecipitation of cell extracts were rinsed twice with Nek2-kinase buffer (50 mM HEPES pH 7.5, 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). Kinase reactions were carried out in 50 μl for 20 min at 30°C in kinase buffer supplemented with 10 μM [^{32}P]γ-ATP (0.2 μCi/μl), 4 μM ATP, 1 μg of cAMP-dependent protein kinase inhibitor, and the appropriate substrate (1 μg of full-length myelin basic protein (MBP), 2 μg of His-HMGA2). Reactions were stopped by adding SDS-sample buffer and analyzed by SDS-PAGE and autoradiography.

Pull-Down Assay

His-HMGA2 (3 μg) were added to 2–4 μg of GST fusion proteins adsorbed on glutathione-agarose (Sigma-Aldrich) in 300 μl (final volume) of PBS with 0.05% BSA, protease inhibitors, and 1 mM DTT for 1 h at 4°C under constant shaking. Beads were washed three times with the same PBS without BSA, eluted in 10 mM glutathione solution. Adsorbed proteins were analyzed by Western blot or Coomassie Brilliant Blue R250 staining.

Electrophoretic Mobility Shift Assay

Gel retardation assay reactions were performed according to Sheflin *et al.* (1993). Briefly, 0.2 pmol of linearized pGEM T easy (Promega, Madison, WI) was mixed to increasing amounts of nonphosphorylated HMGA2[PNek2$_{K37R}$] or phosphorylated HMGA2[PNek2] (from 11 to 120 pmol) in binding buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA, and 0.3 μg/ml BSA, final volume of 20 μl). Mixtures were incubated for 15 min at 37°C, 30 min on ice, and an additional 5 min at 37°C. At the end of the incubation, loading dye was added and DNA–protein complexes were run on 0.7% (wt/vol) agarose gels in Tris-phosphate-EDTA. Electrophoresis was run for 18–20 h at 7.5 V/cm at room temperature, the gel was stained with 0.5 μg/ml ethidium bromide for 1 h, destained in distilled water for 45 min, and photographed.

[^{32}P]Orthophosphate Metabolic Labeling

Isolated spermatocytes were cultured as described above. Cells were preincubated for 12 h with or without the MAPK cascade inhibitor U0126 (Calbiochem) at a concentration of 10 μM. After 12 h, the medium was replaced with phosphate-free minimal essential medium and carrier-free [^{32}P]orthophosphate (0.3 mCi/ml), and spermatocytes were incubated for an additional 2 h. Hence, cells were treated with or without 0.5 μM OA for 6 h. At the end of the incubation, cells were washed three times with PBS, homogenized in Nek2 lysis buffer supplemented with 0.5% Nonidet P-40, and incubated for 15 min on ice. After centrifugation, supernatants were precleared with Sepharose beads for 1 h to reduce the nonspecific binding and then immunoprecipitated for 2 h at 4°C with anti-HMGA2 with new Sepharose beads in the presence of 0.1% BSA. After three washes, the immunoprecipitates were eluted in sample buffer. Samples were separated on a 10% SDS-PAGE, the gel was dried and radioactivity analyzed by autoradiography.

Cross-Linking Experiment

Isolated spermatocytes cultured as described above were treated with either DMSO or 5 μM okadaic acid for 6 h. In the last 10 min of the incubation, cells were cross-linked by adding 1% formaldehyde to the culture medium. Control cells incubated only with DMSO were also collected to monitor the situation of DNA-bound proteins in the absence of cross-link. Cells were collected by centrifugation and resuspended in 1 ml of TRIzol solution (Invitrogen, Carlsbad, CA). RNA, DNA, and protein fraction extraction were carried out following the manufacturer's instruction. DNA fractions resuspended in TE (10 mM Tris and 1 mM EDTA) were treated with 30 μg/ml DNase for 30 min at 37°C and sonicated. DNA and protein fractions were then diluted in SDS-sample buffer for the subsequent Western blot analysis with either anti-Erk1 or anti-HMGA2 antibodies.

RESULTS

HMGA2 Protein Expression in Mouse Germinal Cells

Activation of the MAPK pathway, which leads to phosphorylation and activation of Nek2, is required for chromatin condensation during the G2/M progression of mouse spermatocytes (Sette *et al.*, 1999; Di Agostino *et al.*, 2002). However, so far no chromatin substrates have been identified for the protein kinases involved in this pathway. Because HMGA2 seems to play a crucial role in male fertility but its expression pattern in spermatogenesis is unknown (Chieffi *et al.*, 2002), we set out to determine whether the expression patterns of HMGA2 and Nek2 protein overlap during male germ cell development by using Western blot analysis. HMGA2 protein was expressed in mitotic spermatogonia, meiotic spermatocytes, and postmeiotic round spermatids, whereas it was absent in mature spermatozoa (Figure 1A). Immunofluorescence analysis of testis sections from adult mice indicated that HMGA2 protein mainly localizes to the nuclei of meiotic spermatocytes and postmeiotic round spermatids (Figure 1B). On the other hand, Nek2 expression was limited to meiotic spermatocytes, with only trace amounts detected in mitotic spermatogonia (Figure 1, A and B) (see also Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997; Arama *et al.*, 1998). Thus, HMGA2 and Nek2 are coexpressed in spermatocytes.

Nek2 and HMGA2 Physically Interact

Rhee and Wolgemuth reported that Nek2 associates to condensing chromatin during the prophase of the first meiotic division (Rhee and Wolgemuth, 1997). To test whether Nek2 and the DNA-architectural protein HMGA2 physically interact in mouse spermatocytes, we performed coimmunoprecipitation experiments. Cell extracts from mouse spermatocytes or spermatids were immunoprecipitated using either nonimmune rabbit IgGs or anti-Nek2 IgGs, and the immunopurified proteins were analyzed in Western blot for the presence of HMGA2. As shown in Figure 2A, anti-Nek2 IgGs immunoprecipitated detectable amounts of HMGA2 from spermatocyte extracts where Nek2 is present, but not from spermatid extracts. Furthermore, nonspecific IgGs did not precipitate HMGA2, indicating that the interaction between Nek2 and HMGA2 is specific. When the same samples were stained for p90Rsk2, we found that also p90Rsk2 was coimmunoprecipitated by the Nek2 antibody only from spermatocytes extracts. Interestingly, we observed that Nek2, HMGA2, and p90Rsk2 specifically coimmunoprecipitated from spermatocyte cell extracts also when an anti-HMGA2 antibody was used (Figure 2B). These results suggest that Nek2, its activator p90Rsk2, and the chromatin protein HMGA2 are assembled in a complex in mouse pachytene spermatocytes.

The interaction between Nek2 and HMGA2 is not restricted to meiotic cells, because we observed that recombinant myc-Nek2 could coimmunoprecipitate with recombinant HA-HMGA2 when the two proteins were expressed in the same cells (Figure 2C). Next, we tested whether the interaction with HMGA2 depends on the state of activity of Nek2. A source of active and inactive Nek2 was obtained by

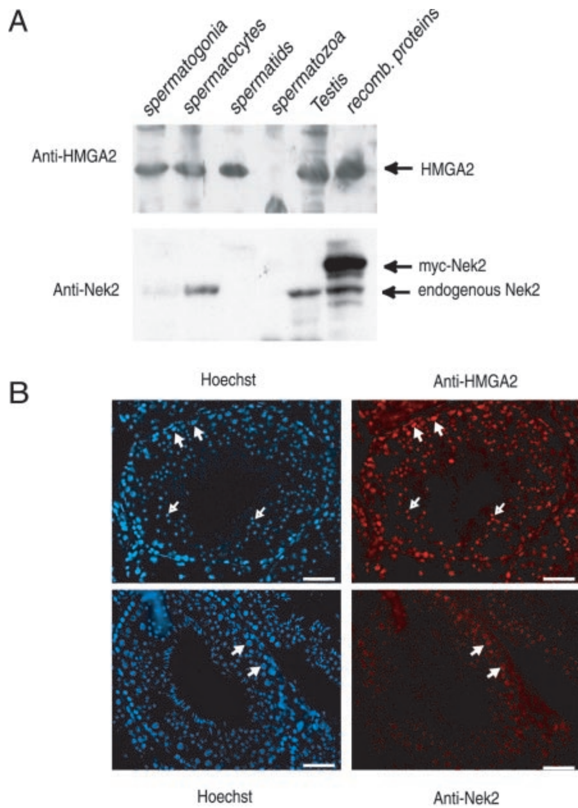

Figure 1. (A) Western blot analyses of HMGA2 and Nek2 in male germ cells. Lysates were prepared from whole testis or from isolated germ cells and separated onto a 10% SDS-PAGE (30 μg for each lane). Western blot analyses were performed with a rabbit polyclonal antibody against HMGA2 (top) or Nek2 (bottom). As positive controls, recombinant purified HMGA2 or cell extracts from HEK293 cells transfected with myc-Nek2 and also expressing endogenous Nek2 were loaded in the rightmost lane of the gels. (B) Immunofluorescence analysis of HMGA2 and Nek2 localization in mouse testis. Sections were fixed and stained with Hoechst (left) and either rabbit anti-HMGA2 antibody or R40 rabbit anti-Nek2 antibody (right). Filled arrows point to two representative meiotic spermatocytes near the periphery of the seminiferous tubule; empty arrows point to two round spermatids near the luminal side of the same tubule.

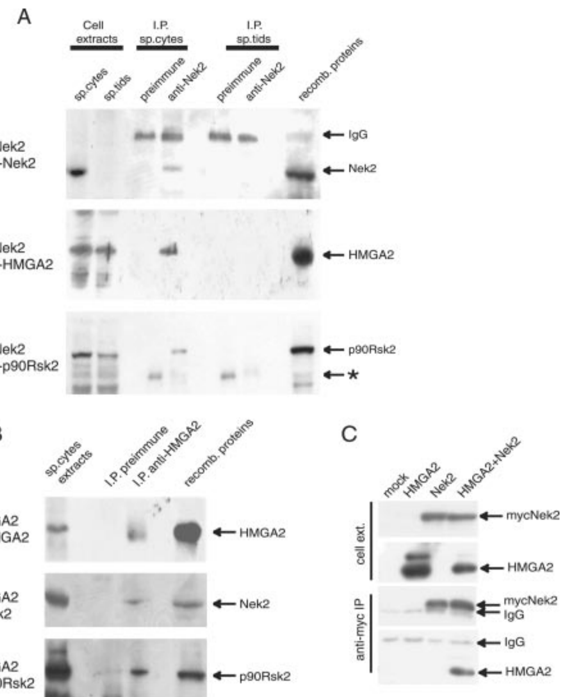

Figure 2. HMGA2 and p90Rsk2 coimmunoprecipitate with Nek2 from mouse spermatocyte extracts. (A) Cell extracts (500 μg) from isolated pachytene spermatocytes or spermatids were immunoprecipitated with polyclonal rabbit preimmune or anti-Nek2 antibody (1 μg). The immunoprecipitated proteins were resolved on SDS-PAGE and subjected to Western blot analysis with anti-Nek2 to assess immunoprecipitation efficiency (top), or anti-HMGA2 (middle), or anti-p90Rsk2 antibodies (bottom), to assess the coimmunoprecipitation of these proteins with Nek2. The asterisk in the bottom panel indicates a nonspecific band recognized by the anti-p90Rsk2 antibody in immunoprecipitates obtained with both preimmune and anti-Nek2 antibody. (B) Spermatocyte cell extracts (500 μg) were immunoprecipitated using 1 μg of either preimmune or anti-HMGA2 antibody. Immunoprecipitated proteins were analyzed in Western blot by using anti-HMGA2 (top), anti-Nek2 (middle), or anti-p90Rsk2 (bottom) antibody. In both A and B, positive controls using either purified (HMGA2, 1 μg) or cell extracts expressing recombinant proteins (for Nek2 and p90Rsk2, 30 μg) were loaded in the rightmost lane of the gels. (C) recombinant HA-HMGA2 and myc-Nek2 were transfected into HEK293 cells in various combinations and cell extracts were immunoprecipitated using the anti-myc antibody. Cell extracts and immunoprecipitated proteins were analyzed in Western blot by using either the anti-myc antibody (first and third panels) or the anti-HA antibody (second and fourth panels).

transfecting Hek293 cells with myc-tagged wild-type or kinase-dead Nek2 and by treating cells for 1 h with okadaic acid to elicit activation of recombinant Nek2 in vivo. Nek2 was immunoprecipitated using the anti-myc antibody and the kinase activity was tested using MBP as substrate. As shown in Figure 3A, okadaic acid strongly stimulated the activity of wild-type Nek2, whereas the effect on kinase-dead $Nek2_{K37N}$ was much less evident. Western blot analysis shows that equal amounts of wild-type or kinase-dead Nek2 were immunoprecipitated by the anti-myc antibody. Next, immunoprecipitated Nek2 was incubated with purified recombinant His-HMGA2 (3 μg). Western blot analysis of the immunocomplexes formed showed that HMGA2 interacted with Nek2 (Figure 3A). Because neither stimulation nor disruption of the catalytic activity of Nek2 affected the binding, the interaction between Nek2 and HMGAs seems to be constitutive. A similar experiment using recombinant HMGA1 gave identical results (our unpublished data), suggesting that Nek2 is capable of interacting with both members of this family.

The Regulatory Domain of Nek2 Directly Binds HMGA2

To test whether the interaction between Nek2 and HMGA2 is direct, we used purified recombinant proteins expressed in *E. coli*. Nek2 is composed of an N-terminal catalytic domain and a C-terminal "regulatory" domain that interacts with the protein phosphatase PP1 (Helps *et al.*, 2000) and the protein kinase Hec1 (Chen *et al.*, 2002), and it is supposed to mediate regulatory events (Fry, 2002). We performed pull-down assays using either GST or GST-$Nek2_{273-444}$, containing the regulatory domain of Nek2. GST proteins were preadsorbed to glutathione-agarose beads and then incubated with 3 μg of purified His-HMGA2 for 90 min. After several washes, the adsorbed proteins were eluted and analyzed in Western blot by using the anti-His antibody. As shown in Figure 3B, GST-$Nek2_{273-444}$ specifically bound to

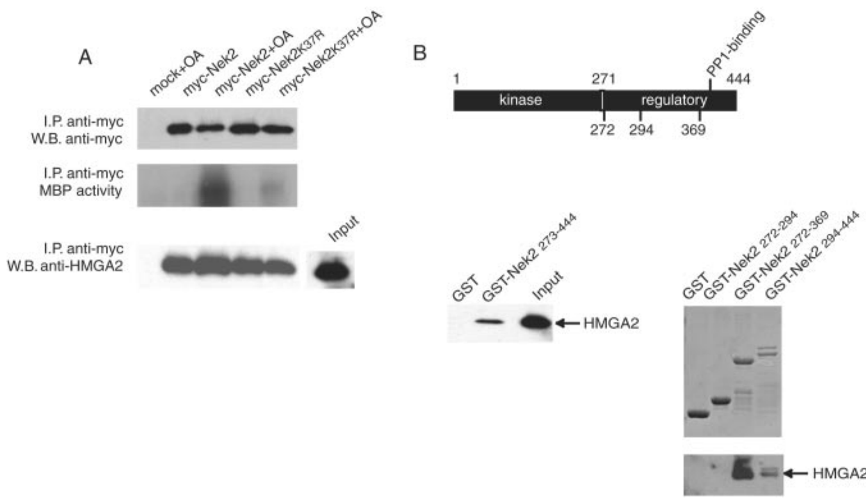

Figure 3. Activation of Nek2 is not required for its interaction with HMGA2. (A) HEK293 cells were transfected with 20 μg of expression vectors for myc-tagged wild-type Nek2 or kinase-dead Nek2K37R. Activation of Nek2 was obtained by treating cells with 0.5 μM OA for the last 2 h before collection. Cell extracts (500 μg) were immunoprecipitated with 1 μg of mouse monoclonal anti-myc antibody (top). Immunoprecipitates were divided in two: half was used for the kinase assay and half for the pull-down assay. Nek2 activity was assayed using MBP (1 μg) and $[^{32}P]\gamma$-ATP as substrates (middle) as described in the MATERIALS AND METHODS. For the pull-down assay, the immunoprecipitates were incubated with 3 μg of purified His-HMGA2, and the immunocomplexes were resolved on SDS-PAGE. Western blot analyses with a mouse monoclonal anti-His antibody were performed to reveal HMGA2. (B) Schematic representation of Nek2: below are indicated the aa selected to separate the three regions of the C-terminal regulatory domain of Nek2 predicted to form α-helices. GST and GST-Nek2 fusion proteins expressed in *E. coli* were adsorbed on glutathione-agarose beads and incubated with purified HMGA2 (2 μg) for 90 min at 4°C. After three washes with PBS, adsorbed proteins were eluted using 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. Eluted proteins were separated on SDS-PAGE and analyzed either by staining the gel with Coomassie Blue (top) or Western blot by using the monoclonal anti-His antibody (bottom).

HMGA2, whereas GST did not; Coomassie staining of the gel demonstrated that the amount of GST proteins used for the pull down was comparable (our unpublished data). Analyses of the Nek2 sequence for structural motifs by using the NPS @nalysis program (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_consensus.pl) indicated that the regulatory domain contains three regions with high probability of α-helix structure: aa 272–286, aa 303–360, and aa 403–430. Thus, we constructed GST-fusion proteins of the regulatory domain to separate these presumptive α-helices: GST-Nek2$_{272–294}$ contains the first helix, GST-Nek2$_{272–369}$ contains the first two, and GST-Nek2$_{294-444}$ contains the second and third helix. Pull-down experiments with purified proteins indicate that HMGA2 preferentially binds to GST-Nek2$_{272–369}$, suggesting that the boundary region between helix 1 and helix 2 of the Nek2 regulatory domain is required for efficient interaction with HMGA2.

Nek2 Phosphorylates HMGA2

To further investigate the role of Nek2 in mouse spermatocyte G2/M progression, we tested whether the kinase was able to phosphorylate its binding partner HMGA2. Indeed, it has been demonstrated that the DNA-binding properties of HMG family members are regulated by posttranslation modifications such as phosphorylation, acetylation, or methylation (Bustin, 1999; Fedele *et al.*, 2001). First, we immunoprecipitated wild-type or kinase-dead myc-Nek2 from control or okadaic acid-treated HEK293 cell extracts and used purified HMGA2 as substrate in immunokinase assays in vitro. As shown in Figure 4A, active Nek2 was able to efficiently phosphorylate HMGA2 whereas the Nek2$_{K37R}$ mutant was not, suggesting that phosphorylation of HMGA2 was indeed due to the immunoprecipitated Nek2. In this experiment, MBP was used as positive control of Nek2 activity.

Next, we tested whether endogenous Nek2 from spermatocytes was also able to phosphorylate HMGA2 in vitro. We isolated Nek2 by immunoprecipitation either from pachytene spermatocytes, in which the kinase is present with low activity, or from metaphase spermatocytes, in which Nek2 has been activated by the MAPK pathway (Di Agostino *et al.*, 2002). Immunoprecipitates were used for

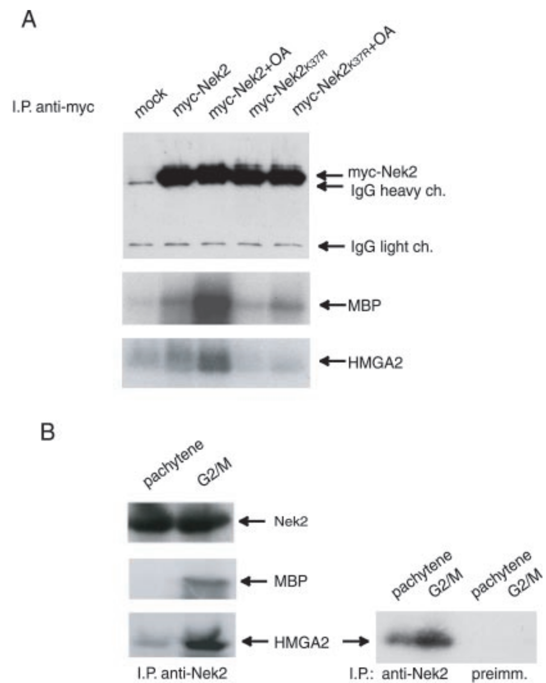

Figure 4. Nek2 phosphorylates HMGA2 in vitro. (A) HEK293 cells were transfected with myc-tagged wild-type Nek2 or kinase-dead Nek2K37R and treated as described in the legend to Figure 3. Cell extracts (500 μg) were immunoprecipitated with 1 μg of mouse monoclonal anti-myc antibody (top). The immunoprecipitates were split in three and used either for Western blot analysis of immunoprecipitated proteins (first panel, position of heavy and light chains of IgGs are indicated by arrows) or for immunokinase assays by using as substrate MBP (2 μg, second panel) as positive control or 2 μg of purified HMGA2 (third panel). (B) Cell extracts (500 μg) from pachytene spermatocytes or spermatocytes induced with OA to progress into metaphase were immunoprecipitated with 1 μg of rabbit polyclonal anti-Nek2 antibody. Nek2 activity in the immunoprecipitates (first panel) was assayed using as substrate 2 μg of MBP (second panel) or HMGA2 (third panel). Mock immunoprecipitation of the same extracts with preimmune IgGs did not have detectable kinase activity toward HMGA2, as shown by the right panel in the figure.

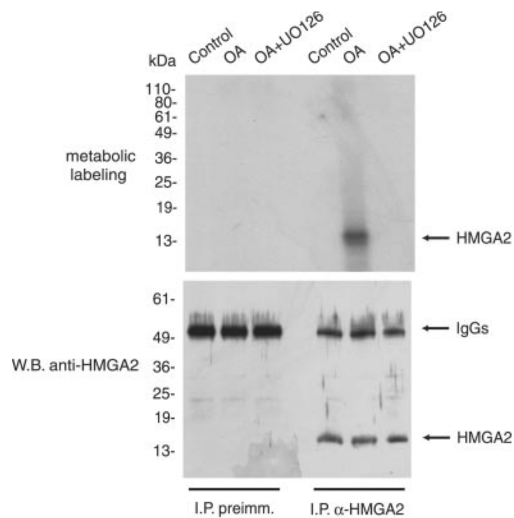

Figure 5. HMGA2 is phosphorylated in an MAPK-dependent manner during the G2/M transition of mouse spermatocytes. Pachytene spermatocytes were labeled with [^{32}P]orthophosphate (0.3 mCi/ml) in phosphate-free medium in the presence or absence of MAPK pathway inhibitor U0126 (10 μM) for 2 h and then treated for 6 h with or without 0.5 μM OA as specified in the figure. Cell lysates were precleared by incubating them for 1 h with protein A-Sepharose beads and then immunoprecipitated with either preimmune serum or anti-HMGA2 antibody. The immunoprecipitated were separated by SDS-PAGE. The gel was either dried and exposed to autoradiography (top) or blotted onto a polyvinylidene difluoride membrane and analyzed in Western blot by using the anti-HMGA2 antibody (bottom).

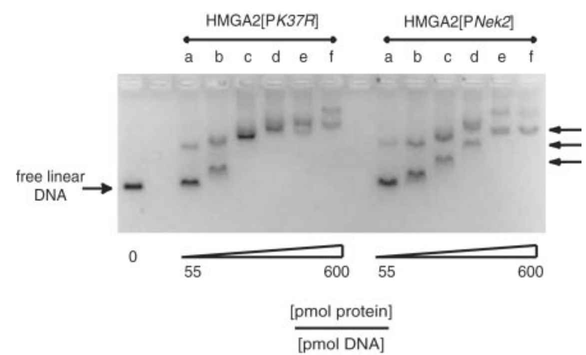

Figure 6. Binding of HMGA2 to linear DNA analyzed by electrophoretic mobility shift assay. Linearized pGEM-T (0.2 pmol, equivalent to ~0.2 μg) was incubated with increasing amounts (11–120 pmol; 0.2–2.3 μg) of HMGA2[P^{Nek2K37R}] and HMGA2[P^{Nek2}] and electrophoresed on 0.7% agarose gel containing 1× Tris-phosphate. DNA was then visualized by ethidium bromide staining. Differences in the mobility of DNA bound to HMGA2[P^{Nek2}] with respect to HMGA2[P^{Nek2K37R}] are indicated by arrows to the right of panels. The molar ratios protein:DNA were a, 55; b, 90; c, 140; d, 230; e, 380; and f, 600.

immunokinase assays by using either MBP, as a control of Nek2 activity, or HMGA2. We found that Nek2 activated during G2/M transition was able to phosphorylate HMGA2 (Figure 4B), whereas only trace amount of kinase activity was immunoprecipitated from pachytene spermatocytes. The kinase activity was due to Nek2 because mock immunoprecipitation of both pachytene and metaphase spermatocyte extracts by using preimmune IgGs did not result in any detectable phosphorylation of HMGA2 (Figure 4B, right).

HMGA2 Phosphorylation in Mouse Spermatocytes

To test whether HMGA2 is phosphorylated during the meiotic progression of mouse spermatocytes, we performed an in vivo metabolic labeling experiment. Pachytene spermatocytes were cultured in phosphate-free medium supplemented with 0.3 mCi/ml [^{32}P]HPO_4 for 2 h in the presence or absence of the MEK1/2 inhibitor U0126 (10 μM) to block the MAPK pathway. Hence, cells were treated with or without 0.5 μM OA to trigger the G2/M progression in the presence or absence of MAPK activation. Cell extracts were prepared from spermatocytes cultured in these different conditions and immunoprecipitation was performed using either nonimmune IgGs or anti-HMGA2 antibody. As shown in Figure 5, OA strongly stimulated the phosphorylation of a protein of the size of HMGA2, which was specifically immunoprecipitated by the anti-HMGA2 antibody. Moreover, phosphorylation of this protein was entirely dependent on the activation of the MAPK pathway. This result suggests that HMGA2 is phosphorylated in vivo during the G2/M progression of mouse spermatocytes by the same pathway required for activation of Nek2 and chromosome condensation.

Phosphorylation by Nek2 Affects the Interaction of HMGA2 with DNA

Previous reports have demonstrated that HMGA1/2 can be phosphorylated by the Cdc2 and casein kinase 2 kinases during mitosis and that these modifications influence the binding of HMGAs to DNA (Wisniewski *et al.*, 1999). Thus, we set out to determine whether phosphorylation by Nek2 affects binding of HMGA2 to DNA. To this end, HMGA2 was phosphorylated in vitro by Nek2 (HMGA2[P^{Nek2}]) and its DNA-binding ability toward linearized plasmid DNA was measured. As control, we used a nonphosphorylated HMGA2 that had been incubated in vitro with kinase-dead $Nek2_{K37R}$ (HMGA2[P^{Nek2K37R}]). Phosphorylated and nonphosphorylated HMGA2 protein were incubated with linearized pGEM-T easy (2.9 kb) at crescent protein:DNA molar ratio and the formation of DNA–protein complexes was monitored by electrophoretic mobility shift assay (Figure 6). As expected, nonphosphorylated HMGA2[P^{Nek2K37R}] was able to bind DNA and cause a shift toward high-molecular-weight complexes in a dose-dependent manner (Figure 6). However, we observed that phosphorylation by wild-type Nek2 modifies the binding ability of HMGA2 and causes the occurrence of a faster complex (arrows in the Figure 6), suggesting that phosphorylation decreases the amount of HMGA2 bound to DNA with the formation of a lighter complex in this in vitro binding assay (Wisniewski *et al.*, 1999).

To test whether the interaction of HMGA2 with DNA was modulated during the meiotic G2/M progression in vivo, we performed a cross-link experiment. Spermatocytes were cultured for 6 h in the absence or presence of okadaic acid to induce transition into metaphase (Wiltshire *et al.*, 1995; Figure 7B), and DNA–protein complexes were fixed by adding 1% formaldehyde in the last 10 min of culture. DNA fraction and protein fraction were isolated from the phenol and chloroform phases obtained with TRIzol lysis of cells and analyzed in Western blot for the presence of HMGA2. As a control, we used pachytene spermatocytes that were not cross-linked with formaldehyde. As shown in Figure 7A (top) in pachytene spermatocytes most of HMGA2 was recovered in the DNA-bound fraction, whereas only trace amount of HMGA2 were detected in the protein fraction. On

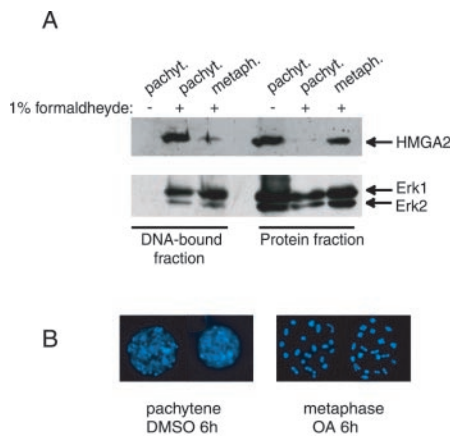

Figure 7. HMGA2 is excluded from chromatin during the G2/M progression of mouse spermatocytes. Mouse spermatocytes (6×10^6 cells) were cultured with or without OA (5 μM) for 6 h to induce a massive G2/M progression. Cells were either collected at the end of the incubation or cross-linked with 1% formaldehyde for 10 min before collection. Cell pellets after centrifugation were resuspended in 1 ml of TRIzol solution and RNA, DNA, and protein fractions were prepared according to manufacturer's instructions. The DNA fraction was treated for 15 min with DNase and sonicated to eliminate excess DNA. Aliquots (15% of total fraction) were analyzed in Western blot by using either the anti-HMGA2 (A, top) or the anti-Erk1 antibody (A, bottom). Representative squashes of nuclei of cells treated with the vehicle DMSO or OA are shown in B: DMSO-treated cells are in the pachytene stage whereas OA-treated cells have entered meiotic metaphase. Nuclei were stained with Hoechst 33342 (1 μg/ml) for 10 min.

the contrary, the pattern of distribution of HMGA2 was completely reversed when cells were in metaphase: HMGA2 was now found mainly in the protein fraction with only trace amounts bound to chromatin. Interestingly, when the same fractions were analyzed for Erk1, which was previously shown to be associated with meiotic chromosomes (Di Agostino *et al.*, 2002), we observed that the MAPK was equally distributed in the protein and DNA fractions and that the cell cycle phase did not affect this distribution dramatically. When cells were not cross-linked, neither HMGA2 nor Erk1 was found in the DNA fraction, indicating that the extraction procedure efficiently separates proteins from DNA. This experiment suggests that phosphorylation of HMGA2 correlates with its exclusion from the chromatin during the G2/M progression of mouse spermatocytes.

DISCUSSION

Nek2 is the mammalian homolog of *Aspergillus nidulans* NIMA kinase, and it plays a role in centrosome duplication during the mitotic cell cycle (Fry *et al.*, 1998a). Nek2 localizes to the centrosome and phosphorylates C-Nap1 (centrosomal Nek2-associated protein), a protein with a coiled-coil structure (Fry *et al.*, 1998b). C-Nap1 links the proximal ends of the duplicated centrioles to each other and its phosphorylation by Nek2 causes loss of centriole-centriole cohesion and centrosome splitting (Mayor *et al.*, 2002).

Nek2 expression and activity peak in the G2 phase of the mitotic cycle and decrease once the cells have entered mitosis due to degradation by the anaphase promoting complex APC^{Cdc20} (Hames *et al.*, 2001). Interestingly, Nek2 is activated in meiosis at a cell cycle stage (G2/M) when it is normally degraded in mitotic cells (Di Agostino *et al.*, 2002); it is possible that during meiosis the APC activator Cdc20 is kept inhibited in the lengthy prophase to delay metaphase and allow completion of meiotic processes such as homologous recombination and homologous chromosome disjunction in diplotene.

The Nek2 homolog NIMA plays a role in chromatin condensation in *Aspergillus nidulans* and in transfected animal cells (Fry, 2002). Several observations suggest that Nek2 could also play such role in meiosis: 1) Nek2 was proposed to associate with condensed chromatin in mouse spermatocytes (Rhee and Wolgemuth, 1997); 2) Nek2 is activated during the G2/M transition of mouse spermatocytes (Rhee and Wolgemuth, 1997; Di Agostino *et al.*, 2002); and 3) Nek2 activation requires the MAPK pathway (Di Agostino *et al.*, 2002), a kinase cascade that promotes chromatin condensation in these cells (Sette *et al.*, 1999). In the present study, we have investigated whether Nek2 directly interacts with DNA or chromatin proteins. We have demonstrated that Nek2 directly associates with the chromatin architectural protein HMGA2 through its C-terminal regulatory domain. Interestingly, it was shown that both Hec1, a protein kinase phosphorylated by Nek2 and required for faithful chromosome segregation (Chen *et al.*, 2002), and PP1, a protein phosphatase that acts in the centrosome duplication pathway like Nek2 (Helps *et al.*, 2000; Eto *et al.*, 2002), bind to this domain of the kinase. Thus, HMGA2 is the third substrate known to interact with the C-terminal regulatory domain of Nek2. However, we found that HMGA2-binding site (aa 272–369) differs from that reported for PP1, (KVHF motif, aa 382–385 in mouse Nek2). Because the regulatory domain of NIMA kinases act as dominant negative in vivo (Lu and Means, 1994), it is likely that these interactions are crucial for the function of Nek2.

Among the known structural chromatin proteins, we have focused our attention on HMGA2 for the following reasons: 1) HMGAs (high-mobility group A proteins) are nonhistone chromosomal proteins that play a role in determining chromatin architecture and in regulating the transcription of several genes (Bustin, 1999; Fedele *et al.*, 2001); 2) HMGA2 is highly expressed in the testis in meiotic and postmeiotic cells (Chieffi *et al.*, 2002; this study); 3) *hmga2*−/− mice are infertile and present degenerating spermatocytes and a drastically reduced number of postmeiotic round spermatids (Chieffi *et al.*, 2002). The HMGA class includes the two isoforms 1 and 2, encoded by the *hmga1* and *hmga2* genes (Bustin, 1999). HMGAs bind DNA in AT-rich regions through three basic domains called "AT-hooks," and they can regulate transcriptional activity by altering the architecture of chromatin and promoting the accessibility to transcriptional factors (Falvo *et al.*, 1995; Foti *et al.*, 2003). Although very similar in function and structure, it was observed that deletion of only one of these genes in mice, *hmga2*, caused a dramatic phenotype called "pygmy," characterized by a great reduction in fat tissue and slow growth due to a longer cell cycle of embryonic fibroblasts (Zhou *et al.*, 1995). Thus, HMGA1 is not able to fully complement HMGA2 function, suggesting that two proteins have specific roles during development. A specific function for HMGA2 should also be involved in the regulation of spermatogenesis, because this differentiation program is dramatically hampered in *hmga2*−/− mice in spite of the presence of HMGA1 (Chieffi *et al.*, 2002). Although we found that Nek2 is able to interact with and phosphorylate both proteins in vitro (Di Agostino and Sette, unpublished observation), it is possible that in vivo, HMGA2 is the more relevant substrate for the kinase or that additional unique roles for HMGAs are exerted during spermatogenesis. Here, we also show that

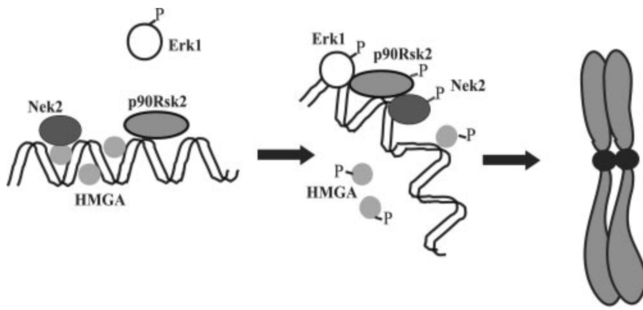

Figure 8. Hypothetical model of the MAPK-dependent stimulation of chromatin condensation in mouse spermatocytes. On activation of the MAPK pathway, p90Rsk2 interacts with and phosphorylates Nek2, which is bound to DNA through interaction with HMGA2. Phosphorylation of HMGA2 by Nek2 decreases its affinity for DNA and favors its release from the chromatin, possibly permitting to condensation factors to enter the chromatin and to trigger chromosome condensation.

the Nek2–HMGA2 interaction is constitutive and independent of the activation state of Nek2. Hence, we suggest that Nek2 might be anchored to chromatin through the association with HMGA2 before it is activated; after stimulation of the MAPK pathway, Nek2 activity increases and HMGA2 is phosphorylated (Figure 8). In agreement with this model is the observation that HMGA2 is phosphorylated in a MAPK-dependent manner in mouse spermatocyte undergoing the G2/M transition. Because the MAPK pathway plays a role in maintaining the chromatin condensed into chromosomes during vertebrate meiosis (Sette *et al*., 1999; Gross *et al*., 2000), it is possible that MAPK-dependent phosphorylation of chromatin molecules, such as HMGA2, is crucial to ensure this process.

We have observed that purified HMGA2 is a good substrate for recombinant and endogenous Nek2 in vitro. Moreover, we found that phosphorylation by Nek2 decreases the affinity of HMGA2 for DNA in gel-retardation assays. Similar results have been reported for mitotic phosphorylation of HMGAs by kinases that are activated at the G2/M transition, such as casein kinase 2 and cdc2 (Piekielko *et al*., 2001). HMG box family proteins are also phosphorylated by mitotic kinases in several organisms (Bustin, 1999). In most cases, it has been shown that phosphorylation decreases the affinity of HMG proteins for DNA in the same electrophoretic assay that we have used in this study. Our data extend this evidence to Nek2-phosphorylation of HMGA2 in mouse spermatocytes and suggest that a reduction of the affinity of HMG proteins for DNA might be a common feature of the entry into metaphase in both mitotic and meiotic cells. In agreement with this hypothesis, we show that upon G2/M transition HMGA2, which is phosphorylated in a MAPK pathway-dependent manner, is excluded from the chromatin.

In conclusion, we have identified a structural chromatin protein as a possible substrate for Nek2 in vivo, which could participate directly or indirectly to chromosome condensation during the G2/M transition of mouse spermatocytes. Because many transcription factors and DNA-repair enzymes are bound to DNA via their interaction with HMGAs (Bustin, 1999; Subramanian and Griffith, 2002), it is possible that Nek2 phosphorylation promotes the release of HMGA2 from the DNA and the turnover of chromatin proteins to allow the entry of other factors involved in condensation of meiotic chromosomes.

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