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Functional Studies of MP62 During Male Chromatin Decondensation in Sea Urchins

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ABSTRACT

In amphibians, sperm histone transition post-fertilization during male pronucleus formation is commanded by histone chaperone Nucleoplasmin (NPM). Here, we report the first studies to analyze the participation of a Nucleoplasmin-like protein on male chromatin remodeling in sea urchins. In this report, we present the molecular characterization of a nucleoplasmin-like protein that is present in non fertilized eggs and early zygotes in sea urchin specie *Tetrapygus niger*. This protein, named MP62 can interact with sperm histones in vitro. By male chromatin decondensation assays and immunodepletion experiments in vitro, we have demonstrated that this protein is responsible for sperm nucleosome disorganization. Furthermore, as amphibian nucleoplasmin MP62 is phosphorylated in vivo immediately post-fertilization and this phosphorylation is dependent on CDK-cyclin activities found after fertilization. As we shown, olomoucine and roscovitine inhibits male nucleosome decondensation, sperm histone replacement in vitro and MP62 phosphorylation in vivo. This is the first report of a nucleoplasmin-like activity in sea urchins participating during male pronucleus formation post-fecundation. J. Cell. Biochem. 114: 1779–1788, 2013.

KEY WORDS: CHROMATIN REMODELING; SPERM HISTONES; MP62; NUCLEOPLASMIN/NUCLEOPHOSMIN; SEA URCHINS; FERTILIZATION

In sea urchins, after fertilization, the male nucleus decondenses and transform into male pronucleus with several biochemical and molecular changes. Specifically, sperm specific histones (SpH) are selectively lost post-fertilization as male chromatin decondenses and fuses with female chromatin, at this moment zygote chromatin is condensed only by maternally inherited histones (Cleavage-Stage histones, CS) [reviewed in Imschenetzky et al., 2003]. Over a decade ago, we have demonstrated that the lost of SpH is commanded by nuclear proteolytic activities found post-fertilization, in this context we have characterized a cysteine protease, named SpH-protease, which specifically degrade male histones leaving CS histones intact [Imschenetzky et al., 1997]. This protease activity is regulated selectively by post-translational modifications present on its substrates: it is inhibited by SpH phosphorylation [Morin et al., 1999a] and poly-ADP ribosylation of CS histones [Morin et al., 1999b]. This proteolytic activity is vital for proper development, since its inhibition by E64d, a general inhibitor of cysteine proteases [Monardes et al., 2005] and the microinjection of antibodies specifically directed against SpH-protease [Puchi et al., 2006] blocks the lost of SpH and initial cell divisions in the zygote. We have demonstrated that SpH-protease is unable to degrade SpH when they are forming nucleosomes. Furthermore, we have described a nucleosome disassembly activity that liberates SpH from male chromatin for proper degradation by SpH-protease [Iribarren et al., 2008].

It is unclear which kind of activity is responsible for SpH liberation from male nucleosomes prior degradation. In batracians, the histones transitions are commanded by Nucleoplasmin. This histone chaperone promotes sperm nucleosome remodeling after fertilization together with N1/N2 complex. In this model, nucleoplasmin assembles nucleosome cores by addition of H2A–H2B dimmers to previously formed H3–H4 tetramers [reviewed by Philpott et al., 2000]. In sea urchins, there has not been characterized a nucleoplasmin-like activity using *Xenopus laevis* nucleoplasmin-isolation and purification protocols [Stephens et al., 2002]. But, with increased amounts in protein sequence data bases, Eirin-Lopez et al.

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[2006] pointed out that a previously isolated protein in sea urchin *Lythechinus pictus* named mitotic apparatus protein (MP62) [Dinsmore et al., 1988] share some nucleoplasmin features, classifying it as a nucleoplasmin-like protein from invertebrates. Based on these antecedents, we have investigated if a putative nucleoplasmin-like activity is present in sea urchins specie *Tetrapygus niger*.

Our studies demonstrate for the first time that MP62 it's a nucleoplasmin family member participating in sperm chromatin remodeling after fertilization and that its activity is modulated by CDK 1–2 dependant phosphorylation during early development in sea urchins.

MATERIALS AND METHODS

GAMETES AND ZYGOTES

Sea urchin *Tetrapygus niger*, were collected from the bay of Concepcion, Chile and maintained in an aquarium containing natural sea water under constant aeration. Unfertilized eggs, sperm and zygotes were obtained as described previously [Imschenetzky et al., 1991].

SUBCELLULAR EXTRACTS

Preparation of S10 cytoplasmic extracts from eggs and zygotes. Non-fertilized eggs and zygotes obtained at different times after fertilization were sedimented at 2,000*g* for 5 min at 4°C. The pellet was washed twice in 10 vol of lysis buffer containing 10 mM HEPES pH 8.0, 250 mM NaCl, 25 mM EGTA, 5 mM $MgCl_2$, 110 mM Glycine, 250 mM Glycerol, 1 mM DTT and 1 mM PMSF. The pellet was resuspended in 1 vol of lysis buffer and homogenized by rapidly aspiration into a 3 ml syringe through a 22 gauge needle and fast expelled back into the tube. This process was repeated until all cells were broken, verified under microscopic observation. Lysate was centrifuged at 10,000*g* for 10 min at 4°C in a microfuge. S10 cytoplasm, that corresponds to a clear part located between the upper lipid layer and the pigmented pellet, was collected and immediately frozen in liquid nitrogen and stored at −80°C.

Nuclear extracts. To obtain the nuclear extracts, unfertilized eggs or zygotes were harvested at different times post-insemination, also homogenized in 10 mM phosphate buffer pH 6.0, 10 mM NaCl, 10 mM EDTA pH 6.0 and 0.5% Triton X-100 and centrifuged at 4,450*g* for 10 min. The pellet containing nuclear proteins was resuspended in 10 mM phosphate buffer pH 6.0, 10 mM NaCl, 10 mM EDTA pH 6.0, total protein was quantified by Bradford method and rapidly frozen in liquid nitrogen and store until use at −80°C.

POLYNUCLEOSOMES ISOLATION

Nuclei were obtained from sperms by an aqueous ethanol/TritonX-100 procedure performed basically as described by Poccia et al. [1981] and modified by Iribarren et al. [2008]. Nucleosomes were obtained by digesting the isolated nuclei with 72 units/ml of micrococcal nuclease (MNase; USBiologicals) in a buffer 0.01 M Tris–HCl at pH 7.6, 0.01 M NaCl, 2.5 mM MgCl2, 1 mM CaCl2 at 37°C for 10 min. The nucleoprotein particles derived from MNase digestion were further purified by centrifugation on a sucrose density gradient 5–20% (w/v) in a buffer 10 mM Tris–HCl pH 7.2 containing 0.7 mM Na_2EDTA and analyzed by electrophoresis on horizontal 1% (w/v) agarose gels in 1 mM EDTA and 0.04 M Tris–acetate buffer pH 8.0, as described by Oliver et al. [2002]. The initial fractions (1–2) of the sucrose gradient containing polynucleosomes free of unbound DNA were used in nucleosome disassembly assays.

SPERM CHROMATIN DISASSEMBLY ASSAY (SCDA)

Polynucleosomes isolated from free DNA by sucrose gradients were used to analyze the potential nucleoplasmin activity of MP62 or the effects of CDK inhibitors Olomoucine and Roscovitine over chromatin disassembly, in vitro. To analyze the SCDA present in the different extracts, 0.5 μg of polynucleosomes were incubated at 37°C with 2 μg of nuclear extracts in a solution containing 10 mM phosphate buffer pH 6.0, 10 mM NaCl, 10 mM EDTA pH 6.0. After incubation, polynucleosome integrity was evaluated by following their electrophoretic migration in 1% (w/v) agarose gels.

Production of recombinant proteins. Recombinant MP62 was produced in *E. coli* BL21(pLys-S) bacterial strain as a (6)-histidine fused protein coded in pRSETB plasmid (Kindly given by Roger Sloboda, Dartmouth University, USA), induction was performed with 1 mM IPTG by 4 h at 37°C. Purification of his-MP62 was made by affinity chromatography with a His-Link Resin (Promega) according to manufacturer's instructions.

IN VIVO PHOSPHORYLATION ASSAYS

In vivo phosphorylation assays were performed according to Monardes et al. [2005] with some modifications. Non fertilized eggs (NFE) were pre incubated with ^{32}P-orthophosphate 20 μCi/ml neutralized with 0.1 N NaOH in sea water by 60 min at room temperature under constant agitation. 30 min pre-insemination, sea urchins NFE were incubated either with olomoucine (5 mM) or roscovitine (0.5 mM) prepared in DMSO. Nuclear extracts were obtained at different times post-fertilization as described above. To select MP62 specifically between nuclear proteins, we had performed immunoprecipitation (IP) experiments using polyclonal MP62 antibodies and protein A-sepharose according to manufacturer's protocols (Invitrogen). As negative control for IP, we used a rabbit non specific IgG. After MP62 IP, radioactivity associated to MP62 phosphorylation was evidenced by autoradiography.

MP62 DEPLETION

To deplete chromatin and S10 cytoplasmic extracts of MP62, we performed tandem immunoprecipitation experiments using polyclonal antibodies against MP62. Briefly, 2 mg/ml of chromatin or S10 extracts obtained at different times after fertilization were incubated 30 min at 4°C with 2 μg of IgG-MP62 and precipitated with protein A-sepharose resin. After a short spin, the resultant supernatant was re-IP as described before two times more to eliminate all endogenous MP62. Presence of MP62 was monitored by western immunoblots with specific antibodies against MP62. MP62-depleted extracts was used immediately or fastly frozen in liquid nitrogen and stored at −80°C.

PREPARATION AND PERMEABILIZATION OF SPERM NUCLEI

All proceedings were performed according the protocols published by Collas and Poccia [1998] with some modifications. 250 μl of

concentrated sperms were suspended in 10 ml of 50 mM HEPES pH 7.3, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine and 0.15 mM spermine (XN buffer) and centrifuged at 2,600*g* for 5 min at 4°C. The nucleus were slightly sonicated and permeabilized in 990 μl of XN buffer and supplemented with 10 μl of ice cold 10% Triton X-100 prepared in XN buffer (0.1% final concentration) this suspension was incubated for 15 min at 4°C under continuous agitation. After centrifugation, sedimented nuclei are washed twice with 1 ml of XN buffer and resuspended in 500 μl of XN buffer to be used immediately or mixed with 500 μl of 100% glycerol/bovine serum albumin 3% (w/v) in 1/1 proportion to be frozen at −80°C for long storage.

MALE PRONUCLEAR FORMATION IN VITRO

One aliquot of demembrated sperm nuclei (aprox. 5×10^7 nuclei/ml) was diluted 20 times in XN buffer. For decondensation reaction, 40 μl of S10 cytoplasmic extract were mixed with an ATP regenerating system (20 mM creatine phosphate, 50 μg/ml creatine kinase (Type I), 2 mM ATP; Sigma Ultragrade) and 4 μl of diluted nuclear suspension. Decondensation is achieved during 1 h of incubation at room temperature and then sperm nuclei were sedimented over 10 mm diameter poly lysine-treated covers slips for 30 s at 4°C. Nuclei were fixed with 3% paraformaldehyde prepared in PBS for 15 min at room temperature, after 3 washes with PBS nuclei were stained with 0.1 μg/ml DAPI for 30 min at room temperature to verify decondensation degree under an epifluorescent microscopy.

Alternatively, for detecting chromatin-bound proteins in this preparation, decondensed male nucleus was underlayed with one volume of 1 M sucrose cushion prepared in SX buffer and centrifuged at 1,000*g* for 30 min at 4°C in a swinging bucket rotor, this proceeding was repeated two times. After this process, chromatin bound proteins were extracted with 2.0 M of NaCl by 2 h at 4°C under constant agitation. This suspension was centrifuged at 30,000*g* for 15 min at 4°C, chromatin-bound proteins in the supernatant were precipitated with 10% of trichloroacetic acid and analyzed by Western blots revealed with antibodies against core SpH.

RESULTS

CHARACTERIZATION OF NUCLEOPLASMIN-LIKE ACTIVITY OF MP62

By amino acid sequence analysis, Eirin-Lopez et al. [2006] report a phylogenetic analysis for nucleoplasmin proteins in several species, in sea urchins a protein named MP62 shares common sequences with invertebrates' nucleoplasmin. To determine if MP62 has a nucleoplasmin-like activity during male pronucleus formation we have performed several experiments to test the capacity of this putative nucleoplasmin protein to decondense sea urchin sperm nucleus in vitro. First, we analyzed if this protein was also present in *Tetrapygus niger* sea urchin. We performed western blot analysis using specific antibodies against *L pictus* MP62. As we show in Figure 1A, our antibodies recognize a single band of 62 kDa in NFE and 10 min zygote whole extracts. To verify the specificity of *L. pictus* antibodies, we performed an immunoabsortion experiment saturating MP62 antibody with an excess of recombinant his-MP62 and tested by western blot in *T. niger* nuclear extract and recombinant his-MP62, as we show in Figure 1B, MP62-specific immuneabsorved antibodies were unable to recognize MP62 in chromatin extracts (lane 2) as also the purified recombinant protein (lane 1). To analyze the presence of MP62 during first embryonic cell cycle, we had performed western blot on cytoplasmic and chromatin extracts (Fig. 1C,D, upper panels). Only one band corresponding to a 62 kDa protein both in cytoplasmic and chromatin extracts in non fertilized eggs and zygotes up to 60 min post-fecundation is present.

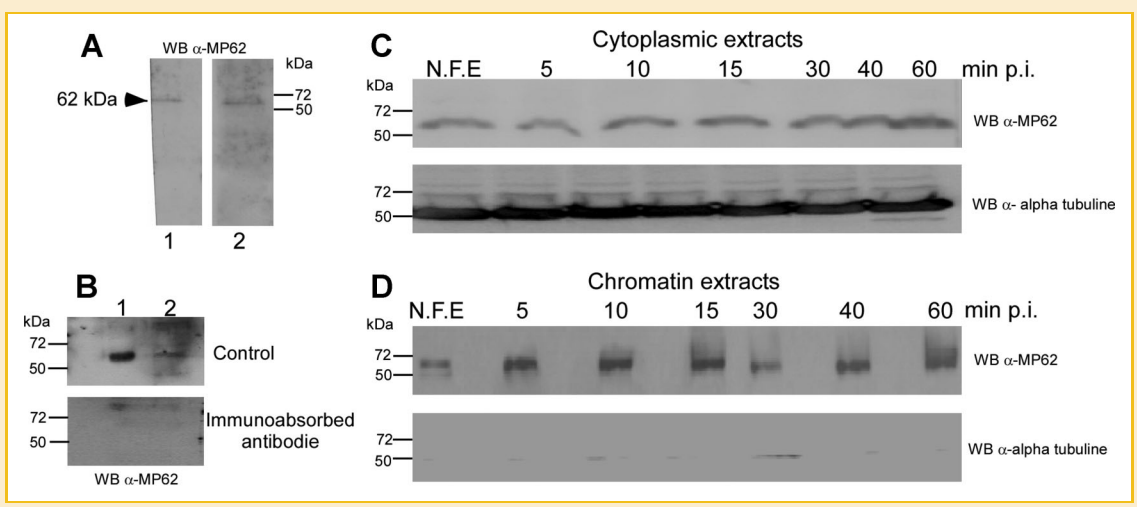

Fig. 1. MP62 identification in sea urchin specie *Tetrapygus niger*. A: Antibodies produced against *Lythechinus pictus* MP62 (LpMP62) were utilized in Western blots assays loading 10 μg of protein from whole extracts from non fertilized eggs (NFE; lane 1) and 10 min p.f. chromatin extracts (lane 2). B: To confirm the crossreaction of LpMP62 antibodies, antibody's immunoabsortion was performed and used in Western blot assay loading 1 μg of his-MP62 (lane 1) and 10 μg of NFE chromatin extract (lane 2). In (C,D), MP62 identification on cytoplasmic (C) and chromatin (D) extracts obtained from NFE and zygotes collected at different times post-fertilization. Alpha tubuline was used as loading and contamination control.

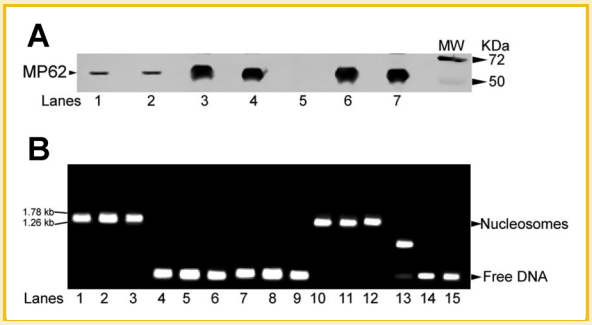

Fig. 2. SCDA experiment utilizing MP62 depleted chromatin extracts. In (A), chromatin extracts obtained 10 min p.f. was MP62 depleted by tandem immunoprecipitation. As explained in Materials and Methods Section, MP62 depletion was performed and monitored by Western blot revealed with specific MP62 antibodies. Lane 1: Chromatin extract 10 min p.f. input, lane 2: mock depleted (non-specific IgG) extract, lanes 3 and 4: 1° and 2° immune complexes from mock depleted extract, lane 5: MP62 depleted chromatin extracts, lanes 6 and 7: 1° and 2° immune complexes from MP62 depleted extract. MW, molecular weight. In (B) sperm polynucleosomes were incubated in each extracts by 0, 15, and 30 min at room temperature, after this, sperm nucleosomes integrity was followed by agarose gel migration. Nucleosomes were incubated in chromatin buffer (lanes 1–3, control), 10 min p.f. zygote chromatin extract (lanes 4–6), mock depleted extract (lanes 7–9), MP62 depleted chromatin extract (lanes 10–12) and suplemented with recombinant his-MP62 into the depleted extract (lanes 13–15).

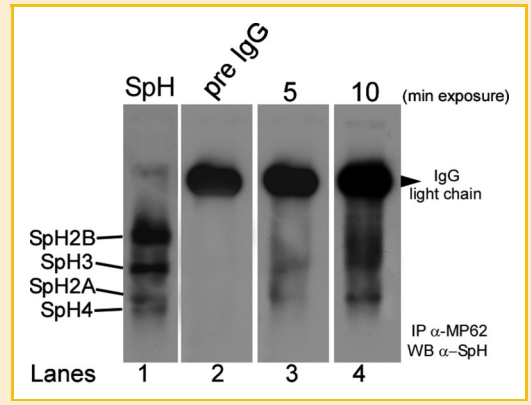

Fig. 3. MP62 interacts with sperm histones in vitro. To determine if MP62 can interact with sperm histones immunoprecipitation experiments on nuclear extracts obtained from zygotes 10 min p.f. with specific antibodies directed against MP62 was performed. Possible interaction was evidenced by SDS–PAGE and western blot revealed with anti SpH antibodies. Lane 1: SpH migration control 0.5 μg, lane 2: preimmune IgG IP negative control, lanes 3 and 4: 5 and 10 min exposure of MP62 IP revealed with anti SpH.

Alpha tubuline was used as cytoplasmic marker and as contamination marker for chromatin extracts (Fig. 1C,D, lower panels).

To demonstrate a possible participation of MP62 during chromatin remodeling, this protein was immunodepleted from chromatin extracts obtained 10 min p.i. by tandem immunoprecipitation experiments with specific antibodies directed to MP62 (Fig. 2A). We confirmed by western blot the complete removal of MP62 from the chromatin extracts, which were used in a sperm chromatin disassembly assay (SCDA; Fig. 2A; lane 5). As shown in Fig. 2B, chromatin extracts obtained 10 min post-insemination completely disassembles sperm chromatin during the time of incubation showing only free DNA (lanes 4–6) as compared with polynucleosomes incubated in control buffer which maintains these migration as discrete particles between 1.26 and 1.78 Kb (lanes 1–3). MP62-depleted chromatin extract was unable to support chromatin decondensation (lanes 10–12), compared with a non depleted extract (lanes 4–6) or mock depleted extract (lanes 7–9). Furthermore, supplementation with recombinant his-MP62 obtained in bacteria, reconstitute chromatine disassembly activity but with some time delay (lanes 13–15).

To explain the participation of MP62 in nucleosome disassembly either a direct or indirect interaction of MP62 and SpH is required. To demonstrate the interaction between MP62 and SpH, we had performed co-immunoprecipitation experiments with specific antibodies against MP62 and nuclear extracts obtained 10 min post-fertilization. Figure 3 shows that MP62 interacts with the whole set of core SpH (SpH2A, SpH2B, SpH3, and SpH4).

Accompanying the morphological changes in sperm nucleus, dramatic changes occur over sperm histones (SpH) content. Immediately post-fecundation, SpH must be released from paternal chromatin and replaced with maternally derived histones. MP62 immunodepletion and in vitro male pronucleus formation using S10 cytoplasmic extracts were carried out to further demonstrate MP62-nucleoplasmin activity. In Figure 4, permeabilized sperm nucleus incubated with control S10 10 min p.i. extracts or mock depleted extract undergoes full chromatin decondensation, viewed as spherical nuclei (Fig. 4B,C) compared with non incubated sperm (Fig. 4A). MP62 depleted extract however was unable to decondense permeabilized nucleus that remained in a conical shape (Fig. 4D), decondensation was recovered after recombinant MP62 was added back into depleted S10 extract (Fig. 4E). Complementing the results, we have analyzed chromatin bound proteins in those remodeled nucleus, Figure 4F (lanes 3 and 4) shows that sperm histones were selectively lost from remodeled chromatin, although in the experiment using MP62-depleted S10 extract, SpH were still present on chromatin (lane 5). This remodeling process was recovered after the addition of his-MP62 into depleted extract (lane 6).

Taken together our results argue for a key role of MP62 as a nucleoplasmin-like protein during sperm remodeling after fertilization, specifically during sperm histones transition.

CDK PARTICIPATION IN SPERM NUCLEOSOMAL DISASSEMBLY

Schnackenberg et al. [2007, 2008] reported the participation of CDK2 during male pronucleus maturation. To further demonstrate a possible role of CDK-dependant phosphorylation during sperm decondensation, we have performed sperm nucleosome decondensation assays with chromatin extracts obtained from zygotes treated with either roscovitine or olomoucine inhibitors of CDK 1 and 2 activities. After incubation of sperm polynucleosomes with those chromatin extracts, we analyzed nucleosome integrity by agarose electrophoresis. The treatment with roscovitine (1 μM; Fig. 5A, lanes

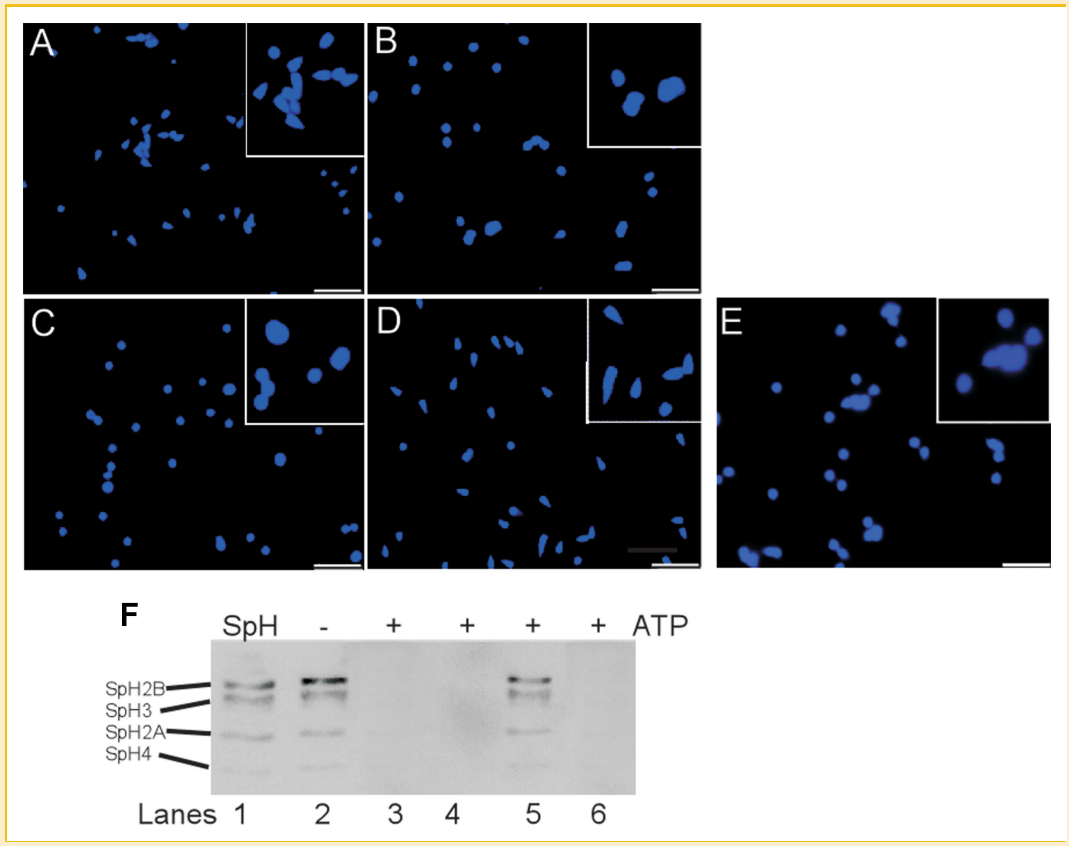

Fig. 4. Male pronucleus formation in vitro. Isolated sperm nucleus was incubated with: S10 10 min p.f. without ATP regeneration system (r.s.; A), S10 10 min p.f. (B), S10 10 min p.f. mock depleted (C) or MP62 depleted (D), recombinant MP62 was added back into depleted S10 extract (E) all incubations were made with an ATP r.s. After decondensation occurs, sperm nuclei was purified, fixed and prepared for nuclear stain with DAPI. Observations were made with an epifluorescent microscopy with 60× magnification. Scale bar 10 μm. In (F) after sperm remodeling in vitro, male nucleus were purified and chromatin bound histones were extracted and precipitated with TCA 10% (v/v). Sperm histones (SpH) transitions were analyzed by Western blot using antibodies directed against sperm core histones. Lane 1: 0.5 μm of core sperm histones was loaded as histone migration control; lane 2: SpH isolated from sperm nucleus incubated with S10 10 min p.f.—ATP r.s.; lane 3: SpH isolated from sperm nucleus incubated with S10 10 min p.f. +ATP r.s.; lane 4: SpH isolated from sperm nucleus incubated with S10 10 min p.f. mock depleted, +ATP r.s.; lane 5: SpH isolated from sperm nucleus incubated with S10 10 min p.f. MP62 depleted, +ATP r.s. Lane 6: SpH isolated from sperm nucleus incubated with S10 10 min p.f. MP62 depleted and suplemented with 0.2 μg his-MP62.

2–5) or olomoucine (10 μM; Fig. 5A, lanes 7–10) blocks normal nucleosomal disassembly compared with non inhibited condition (Fig. 5A, lanes 11–14). This suggests a key participation of CDK activity during sperm nucleosomes decondensation post-fertilization.

Amphibian nucleoplasmin activity is mainly regulated by hyperphosphorylation. Therefore, to study a possible role of CDK-dependant phosphorylation on MP62 during male chromatin remodeling post-fecundation, we performed in vivo incorporation of ^{32}P post-fertilization followed by immunoprecipitation of MP62 (Fig. 5C). As shown in Figure 5B, MP62 was immediately phosphorylated post-fertilization and afterwards their phosphorylation levels decreased in control experiments (compare lanes 4, 7, 10, and 13). This phosphorylation was completely inhibited in 5 min zygotes treated with the CDK inhibitors roscovitine or olomoucine (0.5 or 5 mM each; Fig. 5B: lanes 5 and 6, respectively), at 15, 30, and 60 min p.f. MP62 phosphorylation levels were lower and insensitive to CDK inhibitors (lanes 8, 9, 11, 12, 14, and 15), arguing for participation of another kinase.

Complementing those observations, we used the in vitro sperm remodeling system for reconstitution of some early events of male pronucleus formation, especially morphological changes over chromatin. In this system, CDK participation over male chromatin decondensation was assayed in presence of different concentrations of olomoucine or roscovitine (1–50 μM and 0.1–5.0 μM, respectively). As shown Figure 6B, Olomoucine blocks sperm decondensation at 50 μM and Roscovitine at 1.0 μM, as compared with control experiments showed in Figure 6A, where sperm nucleus were incubated in non treated S10 10 min. p.f. and S10 10 min. plus 0.01% DMSO utilized to prepare CDKs inhibitors (Fig. 6A, lower panels).

To assay SpH replacement and CDK dependant phosphorylation, after sperm nucleus remodeling, we extracted SpH and analyze them by Western blot revealed with SpH core specific antibodies. In Figure 6C, high concentration of olomoucine (50 μM, upper panel, lane 12) impedes sperm histones replacement, this was also evidenced in roscovitine treatment (1 and 5 μM, lower panel, lanes 10 and 12) as compared with sperm nucleus incubated with S10

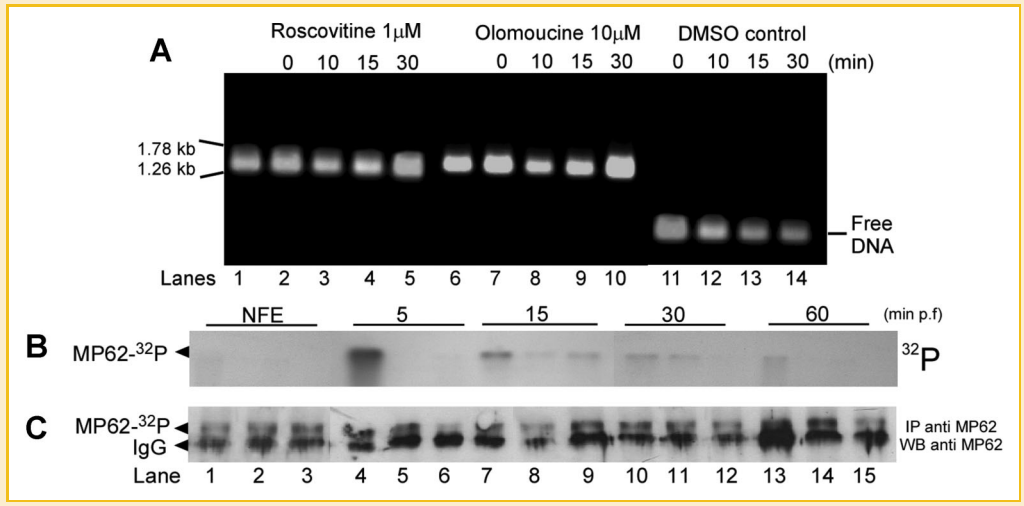

Fig. 5. CDK inhibitors impede sperm nucleosome disassembly in vitro and MP62 phosphorylation in vivo. A: Purified sperm nucleosomes were incubated in chromatin extracts obtained from zygotes 10 min p.f. for 0, 10, 15, and 30 min in presence of roscovitine (1 μM; lanes 2–5), olomoucine (10 μM; lanes 7–10) and DMSO 0,1% (v/v; lanes 11–14), after incubation nucleosomes integrity were analyzed by agarose electrophoresis. Lanes 1 and 6: non incubated nucleosomes. B: Non fertilized eggs (NFE) were pre incubated with 32P-orthophosphate 20 μCi/ml in sea water by 60 min at room temperature. 30 min pre insemination, sea urchins eggs were incubated either with olomoucine (5 mM) or roscovitine (0.5 mM). At different times post-fecundation (p.f.), MP62 was immunoprecipitated (C) from chromatin extracts and phosphorylation was evaluated by incorporation of 32P (B). B,C: lanes 1, 4, 7, 10, and 13: non inhibited condition; lanes 2, 5, 8, 11, and 14: olomoucine treated zygotes, lanes 3, 6, 9, 12, and 15: roscovitine treated zygotes. C: MP62 immunoprecipitation experiment, western blot revealed with antibodies against MP62.

extract (Fig. 6C, lane 4). SpH derived from sperm nucleus incubated in XN buffer (see Materials and Methods Section) was used as migration standard (Fig. 6C, lanes 1 and 2).

Together these results confirm the participation of cyclin dependant kinase activity during morphological and biochemical changes occurring over sperm chromatin after fertilization in sea urchins.

DISCUSSION

Immediately post-fertilization, male nucleus decondenses and it's transformed into the male pronucleus. In sea urchins, this process involves morphological and biochemical changes on male pronucleus chromatin structure. Male chromatin is condensed by a set of sperm specific histones named SpH1, SpH2A, SpH2B, SpH3, and SpH4 (SpH). During male pronucleus formation SpHs are completely lost from male chromatin and replaced with maternally inherited CS. This process involves a specific cystein proteolytic activity which degrades only SpH, named SpH-protease [reviewed by Imschenetzky et al., 2003]. SpH-protease activity is crucial during male histone transitions, since its pharmacologic inhibition or microinjection of SpH-protease specific antibodies in vivo, block normal SpH degradation, male pronucleus formation and microtubular organization, resulting in zygotic abortion [Imschenetzky et al., 1997; Concha et al., 2005].

In this context, we have previously shown that a SpH release from male chromatin is a principal requirement for proper degradation and this activity its present in chromatin extracts from zygotes obtained during male pronucleus formation, but this activity has not been identified [Iribarren et al., 2008].

The main goal of this report was the identification and characterization of this male chromatin remodeling activity. Here, we report that mitotic apparatus-associated protein p62 (MP62) is involved during male pronucleus remodeling, specifically in SpH transitions. We also show that MP62 interacts with SpH, removes them from male chromatin during sperm remodeling and that this activity is dependant on CDK phosphorylation.

MP62 was identified and characterized in sea urchin specie *Lytechinus pictus* by Roger Sloboda group [Dinsmore et al., 1988; Johnston and Sloboda, 1992; Ye and Sloboda, 1995, 1997]. This protein was found as a component of the mitotic spindle during the first cellular division in early development. Molecular characterization and cloning of this protein showed that it has three extremely acid regions rich in glutamic acid (global calculated pI = 4.1), its mRNA is expressed in non fertilized eggs up to gastrula stage, binds to chromatin during interphase and to mitotic apparatus during mitosis [Dinsmore and Sloboda, 1989; Ye and Sloboda, 1995, 1997].

By the other hand, Eirin-Lopez et al. [2006] published an analysis of long-term evolution in nucleoplasmin/nucleophosmin (NPM) family of nuclear chaperones. By sequence analysis of several members of NPM, they showed that MP62 proteins shares some sequence features belonging to invertebrate (equinoderms) NPM in sea urchins species *Lytechinus pictus* and *Strongylocentrotus purpuratus* and star fish specie *Asterina pectinifera*. NPM family of histone chaperones have tree mayor types: NPM1, NPM2, and NPM3.

NPM1 is also known as nucleophosmin, B23, numatrin in mammals and NO38 in amphibians. Localize mainly in the nucleolus in somatic cells and is involved in ribosome biogenesis [Huang et al., 2005], but new roles were reported during cell cycle by binding to

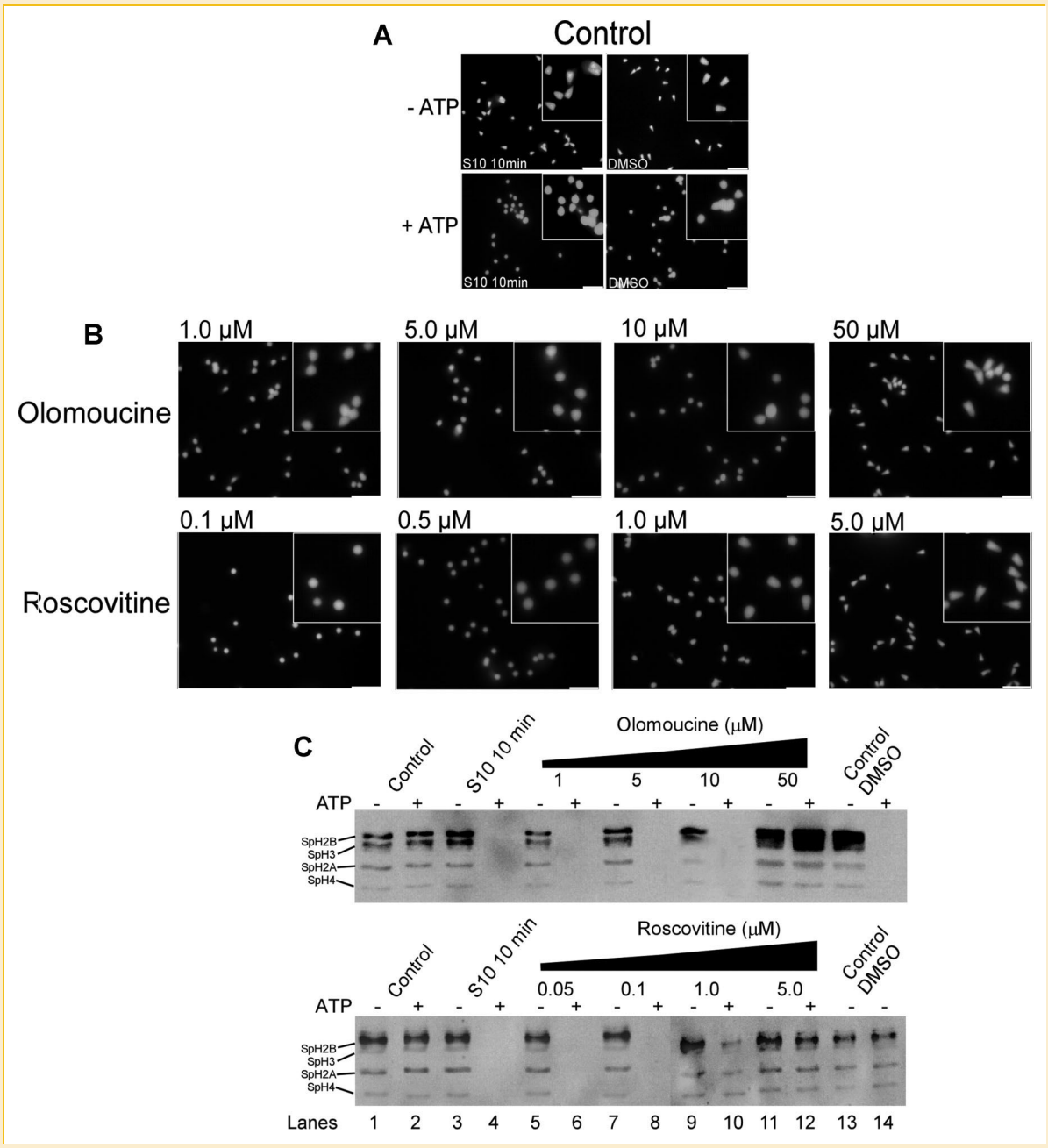

Fig. 6. CDK inhibitors impede male pronucleus formation and sperm histone transitions in vitro. Male pronucleus formation in vitro were carried out to analyze the effect of CDK inhibitor olomuocine and roscovitine on sperm decondensation by nuclear stain with DAPI (A,B) and chromatin bound SpH by Western blot (C). Control decondensation assay are showed in (A), sperm nucleus incubated in S10 10 min p.f. or in presence of 0,1% DMSO, both condition +ATP r.s. (upper panels) or −ATP r.s. (lowers panels). Sperm decondensation in presence of different concentrations of olomoucine (upper panels) or roscovitine (lower panels) are shown in (B). After sperm remodeling, SpH were extracted, precipitated with TCA and analyzed by western blot revealed with anti core SpH (C), sperm nucleus were incubated as follows: lanes 1 and 2: non incubated, lanes 3 and 4: in S10 10 min p.f., lanes 5–12: in S10 10 min p.f. in presence of different concentrations of olomoucine (C, upper panel) or roscovitine (C, lower panel), lanes 13 and 14: incubated in S10 10 min in presence of 0.1% DMSO.

pRb and stimulating DNA pol α [Takemura et al., 1999], centrosome duplication [Okuda et al., 2000] and during transcription by associating to transcription factors and histone chaperone activity [Kondo et al., 1997; Okuwaki et al., 2001; Swaminathan et al., 2005; Weng and Yung, 2005].

NPM2 or nucleoplasmin is the founder member of this nuclear chaperone protein family. It was primarily detected and characterized in *Xenopus laevis* as a major component of chromatin remodeling process during male pronucleus formation [Laskey et al., 1978; Earnshaw et al., 1980; Dingwall and Laskey, 1990]. It is a

thermostable acid protein with a sequence rich in glutamic acid clusters providing binding sites for histones dimers H2A–H2B facilitating their store in oocytes and non fertilized eggs [Dingwall and Laskey, 1990; Philpott et al., 2000; Burns et al., 2003]. In batracians, during male pronucleus formation, sperm specific binding proteins (SSBP) are removed from male chromatin and replaced with H2A–H2B dimers, it was postulated that NPM2 binds to this nuclear proteins and organize their replacement [Ohsumi and Katagiri, 1991; Philpott et al., 1991] together with histone chaperone N1/N2, which binds histone tetramers H3–H4 to assembly new nucleosomes during male pronucleus formation [Dingwall and Laskey, 1990; Philpott et al., 2000].

Xenoupus laevis nucleoplasmin is activated by hyperphosphorylation during oocytes maturation [Leno et al., 1996; Bañuelos et al., 2007]. MP62, like *X. laevis* nucleoplasmin, has several aminoacids residues suitable for CDK phosphorylation. Therefore, a possibility was that CDKs may modulate MP62 activation. Another antecedent is that CDK2-cyclin E activity remains constant over early embryogenesis and moves rapidly into male nucleus post-fertilization and was suggested that this kinase activity is necessary for proper male pronucleus morphological changes during remodeling [Sumerel et al., 2001; Schnackenberg et al., 2007]. In this context, our report demonstrates that MP62 can be phosphorylated immediately post-fertilization and this modification was inhibited by two CDK specific inhibitors, olomoucine and roscovitine. Moreover, sperm nucleosome remodeling and male pronucleus formation was impeded in vitro by these inhibitors. Two key events were blocked: morphological changes on sperm nucleus and sperm histones transition.

In summary, our results demonstrate, for the first time, the presence of a nucleoplasmin-like protein in sea urchin specie *Tetrapygus niger* named MP62 as a principal participant during male pronucleus remodeling post-fertilization, based on our results, we propose the following mechanism for male pronucleus remodeling in sea urchins *T. niger* (Fig. 7).

Male chromatin is compacted by sperm specific histones (SpH, blue) while female chromatin is compacted by poly ADP rybosilated

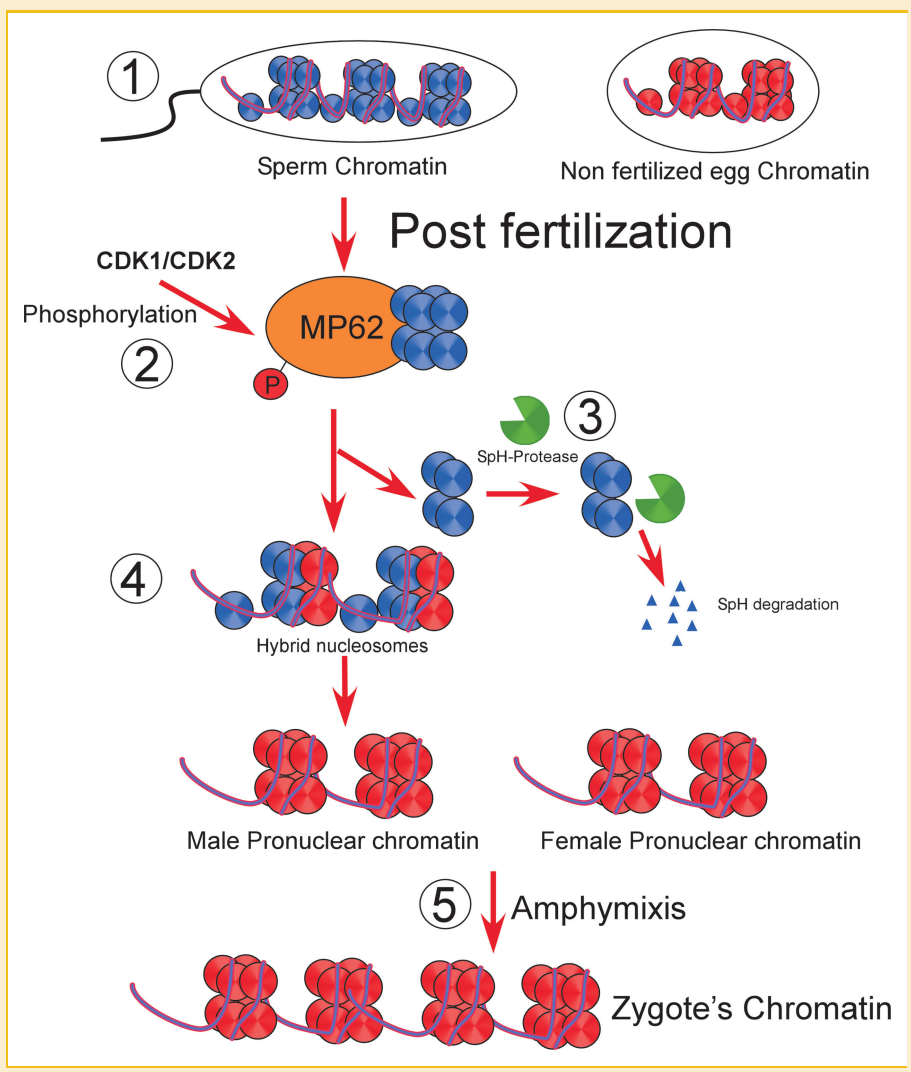

Fig. 7. Model for MP62 participation during male pronucleus formation in sea urchins. See Discussion Section for further details.

histones (CS, red; Step 1). Post-fertilization, MP62 migrates to the male nucleus and is phosphorylated by CDK1/2-dependant activity (Step 2). In this context, we had shown that MP62 interact with whole set of sperm histones in vitro, but it is very interesting if CDK-dependant phosphorylation influences MP62 interaction and removal of sperm histones from male nucleosomes and mediates specific SpH-protease degradation of them; which is a key event in male pronucleus formation (Step 3), those mechanistic issues deserves future analysis. In an intermediate state of male pronucleus formation, hybrid nucleosomes constituted by a set of SpH and CS histones have been identified (Step 4). Finally, both male and female pronuclei are compacted only by CS histones and fuses to restore the diploid condition of the new embryo (Step 5).

MP62 has a key role on sperm nucleosome disassembly in vitro and in vivo, since MP62 depleted extracts were unable to decondense sperm chromatin, interact with core sperm histones and it is necessary for proper male pronucleus formation. We have also demonstrated that MP62 activity can be regulated by CDK-phosphorylation. In fact, MP62 is phosphorylated by a CDK activity immediately post-fertilization in vivo and this activity modulate sperm nucleosome disassembly, morphological decondensation events and sperm histone transitions in vitro.

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