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An Intact Sperm Nuclear Matrix May Be Necessary for the Mouse Paternal Genome to Participate in Embryonic Development[1]

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ABSTRACT

We have been interested in determining the minimally required elements in the sperm head that are necessary in order for the paternal genome to participate in embryogenesis. We used an ionic detergent, mixed alkyltrimethylammonium bromide (ATAB), plus dithiothreitol (DTT) to remove the acrosome and almost all of the perinuclear theca, leaving only the sperm nucleus morphologically intact. We also tested the stability of the sperm nuclear matrix by the ability to form nuclear halos. Sperm nuclei washed in freshly prepared 0.5% ATAB + 2 mM DTT completely decondensed when extracted with salt, but nuclei washed in the same buffer that was 1 wk old, and then extracted with salt, produced nuclear halos, indicating stable nuclear matrices. When we treated sperm heads with freshly prepared ATAB+DTT and injected them into oocytes, none of the oocytes developed into live offspring. In contrast, sperm heads treated in the same way but with 1-wk-old ATAB+DTT solution could support development of about 30% of the oocytes to live offspring. Electron microscopy demonstrated that most of the perinuclear theca had been removed in both cases. These data suggest that at least in the mouse, the only component of the spermatozoa that is crucial for participation in embryologic development is the sperm nucleus with a stable nuclear matrix.

INTRODUCTION

We are interested in identifying the minimal components of the sperm head that are essential for allowing the paternal genome to participate in embryonic development. An important question in this research is the role of the complex structural organization of the sperm DNA in embryogenesis. Mammalian sperm DNA is organized into loop domains that are attached at specific sites to the structural component of the nucleus, the sperm nuclear matrix [1–3]. DNA loop domain organization has been shown to be involved in both DNA replication [4] and transcriptional regulation [5]. Moreover, this type of chromatin structure varies significantly in different cell types of the same organism [1, 2]. It is reasonable to predict that such a complex structural organization in the sperm nucleus may contribute to the participation of the paternal genome in embryonic development.

The sperm head can be used for intracytoplasmic sperm injection (ICSI) to produce viable offspring, but exactly what elements are required for embryogenesis is still not understood. The sperm head is a complicated structure, containing not only the sperm nucleus with all the paternal DNA but many other cellular elements. These include the plasma membrane, the acrosome and its contents, the acrosomal matrix, and the perinuclear theca, a cytoskeletal structure that completely envelops the nucleus and the nuclear envelope [6–9]. It is unclear whether mouse sperm nuclei that lack all of these structures can be used for ICSI to produce fertile offspring [10, 11].

In the present study we used an ionic detergent, mixed alkyltrimethylammonium bromide (ATAB), to completely remove all cytoplasmic elements from the sperm head, leaving only the sperm nucleus intact [12, 13]. We also examined the structural organization of the DNA within the sperm nucleus to determine whether there was a correlation between nuclear structure and the production of viable offspring.

MATERIALS AND METHODS

Treatment of Mouse Spermatozoa with ATAB

The epididymal spermatozoa from one mouse ($B6D_2$ F1) were collected and then suspended in 2 ml of nuclear isolation medium (NIM: 121.6 mM KCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1% polyvinyl alcohol, 10 mM EDTA, 0.5 mM PMSF; all supplied by Sigma Chemical Co., St. Louis, MO) supplemented with an ionic detergent, 0.5% mixed ATAB (Sigma), and 2 mM dithiothreitol (DTT; Sigma); the pH was adjusted to 8.2 by adding drops of 1 M KOH. This suspension was either used fresh or used after 1 or 2 wk of storage at 4°C, during which time most of the DTT was oxidized (see *Results*). ATAB separates the heads from the tails in both cases. Spermatozoa suspended in this medium for 5–15 min at room temperature were occasionally vortexed, then centrifuged at 700 × *g* for 5 min. The sperm pellet was washed in 10 ml of NIM without ATAB or DTT and centrifuged again. The pellet was resuspended in NIM with 6% (w:v) polyvinylpyrrolidone (M_r 360 000; ICN Pharmaceuticals, Irvine, CA), and a single sperm head was used for ICSI.

Examination of Nuclear Structure

The epididymal spermatozoa from one mouse were collected and suspended in 2 ml of NIM containing ATAB and DTT (NIM-ATAB/DTT), either fresh or 1 wk old, and then washed with 2 ml of NIM as described above. The pelleted nuclei were resuspended in 100 μl of 2 M NaCl, 25 mM Tris, pH 7.4, with or without 2 mM DTT. DTT in this salt buffer was found to be necessary to extract the protamines from sperm nuclei when 1-wk-old buffer was used, but not from freshly prepared NIM-ATAB/DTT. This suggested that DTT was oxidized during the week-long storage, and the experiments were repeated using NIM containing only ATAB (NIM-ATAB) with and without 2 mM DTT. The nuclei were incubated in the salt extraction buffer

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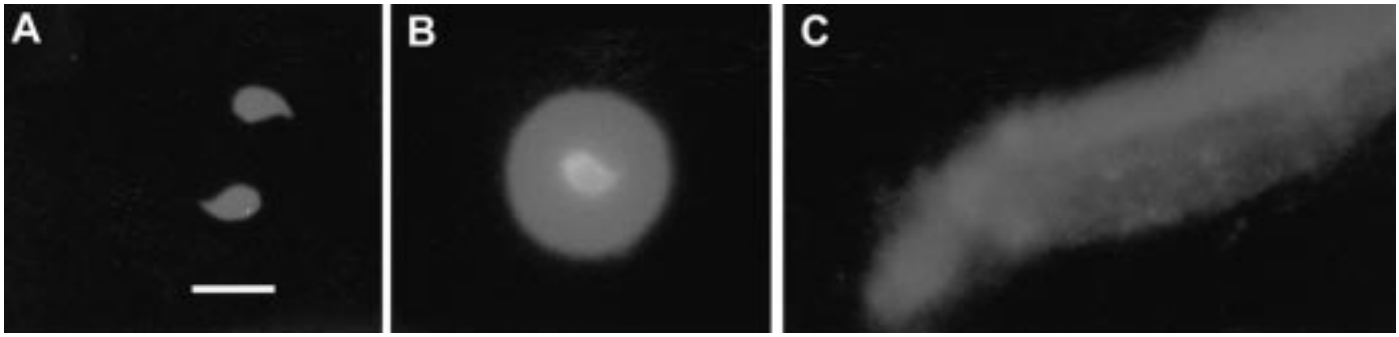

FIG. 1. DNA organization in mouse sperm nuclei treated with ATAB. Mouse sperm nuclei washed with ATAB under various conditions were subsequently extracted with 2 M NaCl to remove the protamines and were stained with ethidium bromide to visualize the DNA. **A**) Condensed: A condensed mouse sperm nucleus from which the protamines were not extracted. This was typical of spermatozoa treated with 1-wk-old NIM-ATAB/DTT or with fresh NIM-ATAB and extracted with 2 M NaCl without additional DTT. **B**) Nuclear halo: This nucleus was treated as in **A** except that 2 mM DTT was included in the high-salt protamine extraction buffer. The nuclear matrix remained intact, and the DNA appears as a halo of fluorescence surrounding the nucleus. **C**) Decondensed: This nucleus was treated with fresh NIM-ATAB/DTT and completely decondensed when subsequently extracted with salt. No nuclear structure remained in these preparations. Treatment with 1-wk-old NIM-ATAB/DTT with freshly added DTT gave the same result. See Table 1 for results of all treatments. All micrographs are shown at the same magnification; bar = 10 μm).

for 10 min on ice and then stained with 100 μg/ml ethidium bromide to detect the DNA.

Preparation of Oocytes

B6D2F1 females, 8–10 wk, were induced to superovulate by consecutive injections of 5 IU eCG and 5 IU hCG 48 h apart. Oocytes, collected from oviducts 14–15 h after hCG injection, were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (300 USP U/ng; ICN Pharmaceuticals) in Hepes-buffered CZB medium (Hepes-CZB) [14]. The oocytes were washed thoroughly and kept in CZB for up to 2 h at 37°C under 5% CO_2 in air. Oocytes to be injected with spermatozoa were transferred into a droplet (6 μl) of Hepes-CBZ under mineral oil (Squibb and Sons, Princeton, NJ) in an operation dish on the microscope stage [14].

Sperm Injection

Sperm nuclei treated with ATAB+DTT were washed in Hepes-CZB containing 6% polyvinylpyrrolidone immediately before ICSI. A single sperm nucleus was aspirated into the injection pipette attached to the micromanipulator and injected into an oocyte according to the method described by Kimura and Yanagimachi [14] except that all operations were carried out at room temperature (about 25°C) instead of 16–17°C. Approximately 10 min after sperm injection, the oocytes were transferred into Ca^{2+}-free CZB containing 5 mM $SrCl_2$ and incubated for 1 h to activate them [15]. Unlike fresh spermatozoa [14], sperm nuclei washed with ATAB+DTT could not activate oocytes when injected into them, so incubation in $SrCl_2$ was necessary to activate the oocytes. Sperm-injected oocytes were incubated for about 5 h in CZB medium and examined with an inverted microscope. Those with two distinct pronuclei and the second polar body were considered normally fertilized.

Embryo Transfer to Foster Mothers

Two-cell embryos arising from normally fertilized eggs were transferred to oviducts of ICR (albino) female mice that had mated with vasectomized males of the same strain during the previous night [16]. Females were allowed to deliver and raise their foster pups (black eyes and pigmented coats).

Electron Microscopy

Sperm heads were fixed in 2% glutaraldehyde in cacodylate buffer, embedded in Spurr (Ladd Research Industries, Burlington, VT), and thin-sectioned for transmission electron microscopy.

RESULTS

Sperm Nuclear Matrix Stability of ATAB-Washed Spermatozoa

Several groups have suggested that chromatin structure may play an important role in embryogenesis [17, 18]. We therefore tested the hypothesis that a stable nuclear matrix may contribute to the participation of the paternal genome in embryogenesis. We developed treatments under which the mouse sperm nuclear matrix was either stable or unstable using very similar buffer conditions. In this manner, these different treatments of mouse sperm nuclei used for ICSI could be interpreted more accurately.

The test we used for nuclear matrix stability was to extract treated nuclei with 2 M NaCl and 2 mM DTT to remove the protamines. The nuclear structures that resulted were examined by staining with ethidium bromide under fluorescence microscopy. Sperm nuclei with stable nuclear matrices will form nuclear halos under these conditions [1, 19]. Nuclear halos are prepared from nuclei by extracting the protamines with salt buffer. The nuclear matrix, the skeletal component of the sperm nucleus, retains the original hooked shape of the nucleus, and the DNA emanates from the nuclear matrix in the form of loops, creating a halo of DNA surrounding the nuclear matrix [1, 19].

We found that the nuclei of spermatozoa washed with NIM-ATAB without DTT formed nuclear halos when extracted with 2 M NaCl + 2 mM DTT, indicating that this treatment resulted in sperm nuclei with stable nuclear matrices (Fig. 1B). However, for spermatozoa treated with NIM-ATAB/DTT, the nuclear matrices completely decondensed when extraction was carried out with NaCl+DTT (Fig. 1C). Surprisingly, when NIM-ATAB/DTT was prepared and incubated at 4°C for 1 wk, the nuclei formed nuclear halos when extracted with 2 M NaCl+DTT, indicating that the nuclear matrices were stable (Table 1). These data suggested to us that treating mouse spermatozoa with 0.5% ATAB in the presence of 2 mM DTT destabilized the nuclear matrix, but only when the buffer was freshly pre-

TABLE 1. Sperm DNA organization by the sperm nucleus, examined after sperm heads were washed in the initial buffer, then extracted with 2 M NaCl.

Initial buffer	DTT in 2 M NaCl	DNA organization	Nuclear matrix stability
Fresh NIM-ATAB/DTT	No	Decondensed	Unstable
Fresh NIM-ATAB/DTT	Yes	Decondensed	Unstable
Fresh NIM-ATAB	No	Condensed	Stable
Fresh NIM-ATAB	Yes	Nuclear halos	Stable
1-Wk-old NIM-ATAB/DTT	No	Condensed	Stable
1-Wk-old NIM-ATAB/DTT	Yes	Nuclear halos	Stable
1-Wk-old NIM-ATAB/DTT + fresh DTT	No	Decondensed	Unstable
1-Wk-old NIM-ATAB/DTT + fresh DTT	Yes	Decondensed	Unstable

pared. To ensure that this impressive difference in nuclear structure seen after storage for 1 wk was due only to the presence of DTT in the NIM-ATAB/DTT extraction buffer, we repeated the experiment using 1-wk-old NIM-ATAB/DTT to which additional DTT was added on the day of the experiment. These nuclei decondensed in the presence of 2 M NaCl. The results were consistent with the conclusion that DTT in the presence of ATAB was destabilizing the nuclear matrix (Table 1).

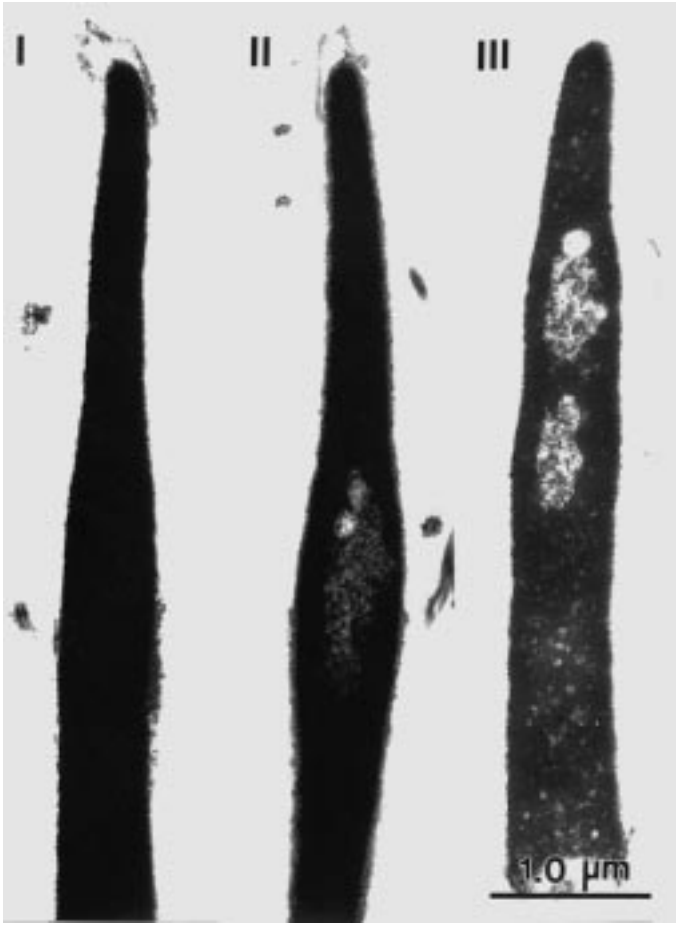

FIG. 2. Electron micrographs of ATAB-treated mouse sperm nuclei. Three different levels of chromatin decondensation were noted when sperm nuclei were treated with ATAB in the presence or absence of DTT. (I) Type I nuclei were intact and fully condensed; (II) Type II nuclei had some areas in the central portion of the nucleus that appeared to be slightly decondensed; and (III) Type III showed large areas of the nuclei that contained an open chromatin configuration. The results for each treatment are shown in Table 2. Note that in all three types, the perinuclear theca has been largely removed.

Evidence That DTT Loses Most of Its Disulfide-Reducing Ability after One Week

The nuclear matrix stabilization assay (Fig. 1, Table 1) data suggested to us that the DTT lost its ability to reduce disulfides during the 1 wk of storage in NIM-ATAB/DTT. To test this hypothesis we examined the ability of 2 M NaCl alone to extract the protamines from the treated mouse sperm nuclei. Hamster [19], human [20], and mouse (unpublished results) sperm nuclei either decondense completely or form nuclear halo structures when the protamines are extracted with 2 M NaCl in the presence of DTT. Without DTT, 2 M NaCl cannot extract the protamines from mammalian sperm nuclei since they are cross-linked by disulfide bonds [21]. When mouse spermatozoa were treated with freshly prepared NIM-ATAB/DTT and then extracted with 2 M NaCl in the absence of additional DTT, the nuclei decondensed completely (Fig. 1C, Table 1). This suggested that the DTT in the fresh NIM-ATAB/DTT was still capable of reducing the intramolecular disulfide bonds between the protamines. When the experiment was repeated with 1-wk-old NIM-ATAB/DTT, however, the nuclei remained condensed, indicating no evidence of protamine extraction (Fig. 1A, Table 1). This suggested that after 1 wk of storage the DTT had lost its ability to reduce the protamine disulfide bonds. When DTT was later added to the 2 M NaCl extraction buffer as a control, the nuclear halos did form, indicating successful extraction of the protamines (Table 1).

Electron Microscopy of ATAB-Treated Spermatozoa

Ultrastructural examination of sperm nuclei showed that most of the perinuclear theca was removed by treatment with NIM-ATAB, with or without DTT. After exposure to NIM-ATAB (without DTT), sperm chromatin remained condensed and uniformly electron dense (Fig. 2, Type I nuclei, and Table 2). ATAB treatment in the presence of DTT resulted in pronounced foci of decondensed chromatin in the center of most of the nuclei (Fig. 2, Type III nuclei, and Table 2), although they appeared intact when visualized by light microscopy. However, when the NIM-ATAB/DTT was allowed to age for 1 wk before use, this ultrastructural change was less extensive and was seen to have occurred in fewer sperm nuclei (Fig. 2, Types II and III, and Table 2).

Development of Oocytes Injected with ATAB-Treated Sperm Nuclei

The data from the nuclear matrix stabilization assay (Fig. 1 and Table 1) suggested that the nuclei from spermatozoa washed with freshly prepared NIM-ATAB/DTT were not stable, but nuclei washed in the same buffer that was stored at 4°C for 1 wk contained stable nuclear matrices. The ev-

TABLE 2. Analysis of nuclear morphology following treatment of sperm nuclei with ATAB in various conditions.

Treatment	Nuclear morphology by electron microscopy (%)		
	Type I	Type II	Type III
Fresh NIM-ATAB/DTT	0	11	89
Fresh NIM-ATAB	100	0	0
1-Wk-old NIM-ATAB/DTT	31	31	38
1-Wk-old NIM-ATAB/DTT + Fresh DTT	3	19	78

TABLE 3. Development of oocytes to pronuclear stage after sperm nuclei, washed with both fresh and old NIM-ATAB/DTT, were injected into oocytes.

Treatment	No. oocytes injected	No. oocytes survived	No. (%) activated eggs with 2 pronuclei and 2nd polar body
Fresh NIM-DTT/ATAB, 5 min	88	71	54 (76)
1-Week-old NIM-DTT/ATAB 15 min	78	67	62 (93)

TABLE 4. Development of embryos to live offspring, after sperm nuclei, washed in both fresh and old NIM-ATAB/DTT, were injected into oocytes.

Treatment	No. embryos transferred	No. recipients	No. (%) of live offspring
Fresh NIM-ATAB/DTT, 5 min	49	4	0 (0)
1-Wk-old NIM-ATAB/DTT, 15 min	63	5	19 (30)

idence suggested that the DTT had lost most of its ability to reduce the protamine disulfide bonds during this storage, even though electron microscopy suggested that the buffer contained some reducing ability. This gave us a method to treat spermatozoa using the same buffer that resulted in spermatozoa with different nuclear matrix stabilities.

Mouse oocytes injected with sperm nuclei that were treated with freshly prepared or 1-wk-old NIM-ATAB/DTT both developed to the pronuclear stage (Table 3). There was a slight decrease in the number of oocytes that developed to this stage using fresh buffer, 76% compared to 93% for the week-old buffer. However, only those nuclei that were treated with 1-wk-old NIM-ATAB/DTT, which contained stable nuclear matrices, had the ability to participate fully in embryogenesis after ICSI (Table 4).

DISCUSSION

One goal of these experiments was to test the prediction that the sperm nucleus—devoid of all the cytoplasmic elements with which it is closely associated—is all that is necessary for the paternal genome to participate fully in embryogenesis. Previous experiments had demonstrated that sperm heads that had no traces of the acrosome, acrosomal matrix, plasma, or nuclear membranes, and had only a partial perinuclear theca, were fully capable of normal embryogenesis when used in ICSI [10]. In these experiments, ATAB was used to completely remove the perinuclear theca [13]. Bellve and colleagues [6] used a similar detergent, CTAB (cetyltrimethylammonium bromide), to effect complete removal of the perinuclear theca from mouse spermatozoa. In our studies, electron microscopy was used to demonstrate that under all conditions used, the perinuclear theca was absent. Thus, we demonstrated that the mouse sperm nucleus is the only component of the spermatozoa that is required for embryonic development when used with ICSI.

We also found that only sperm nuclei treated with NIM-ATAB/DTT that had been stored for 1 wk were capable of participating in embryogenesis when used for ICSI. Nuclei treated with fresh NIM-ATAB/DTT were not. Two lines of evidence suggested that the major difference between the freshly prepared and 1-wk-old NIM-ATAB/DTT was that in the latter, the DTT was oxidized. First, electron micrographs indicated that sperm nuclei treated with fresh NIM-ATAB/DTT were slightly decondensed, while those that were treated with week-old buffer were not. Second, 2 M NaCl was capable of extracting the protamines from sperm nuclei treated with fresh NIM-ATAB/DTT without additional DTT, while those washed with week-old buffer required additional DTT. In both treatments, however, the perinuclear theca was removed.

The data suggested that the presence of DTT in the initial ATAB extraction buffer could destroy the ability of the sperm nucleus to participate in embryogenesis, but the reason for this was not immediately clear. DTT would not be expected to damage the DNA directly, and it is unlikely that the sperm nucleus would contain any DNases that were activated by DTT (the only other explanation for DNA damage we could identify). We therefore examined the possibility that DTT in the presence of ATAB was destabilizing the nuclear matrix and that this destabilization is what was important for inhibiting embryogenesis. We have previously demonstrated that the sperm DNA contains a complex organization and have suggested that this organization may be important for embryogenesis [1, 17, 20]. Sperm DNA is organized into loop domains that are attached by specific sequences to the structural component of the nucleus, the sperm nuclear matrix, and this organization is different from that of somatic cells [1–3, 17]. This DNA loop structure is known to be important for DNA replication [4] and for gene regulation [5], so it is possible that sperm DNA loop domain organization plays a role in the regulation of the paternal genome during embryogenesis.

The data in this work suggest, though they do not yet prove, that this may be the case. We found that sperm nuclei treated with freshly prepared NIM-ATAB/DTT completely decondensed when extracted with 2 M NaCl, but that NIM-ATAB with no DTT (or week-old NIM-ATAB/DTT) formed nuclear halos when extracted with salt and DTT. This suggested that the nuclear matrices and the overall DNA loop domain organization was much more stable in those nuclei treated with ATAB alone. Those nuclei that

were still capable of forming nuclear halos when the protamines were extracted could also participate in embryogenesis (e.g., week-old NIM-ATAB/DTT), but those that decondensed when the protamines were extracted could not (Table 4). It is possible that washing sperm nuclei with ATAB in the presence of DTT also affects other aspects of sperm chromatin structure that are also necessary for embryonic development, and further experimentation will be necessary to identify these. It is clear, however, from these experiments that the nuclear matrix is destabilized by this treatment and that these nuclei cannot participate in embryonic development.

It is very clear from many previous experiments that DTT in the absence of any ionic detergent does not destabilize the nuclear matrix [1, 17]. In fact, DTT is necessary to extract the protamines so that nuclear halos can be formed. The data in this work demonstrate that it is only when DTT is included in the presence of ATAB, an ionic detergent, that the nuclear matrix is destabilized. There are at least two possible mechanisms for this. The first is that since the nuclei treated with fresh NIM-ATAB/DTT are slightly decondensed, it is probable that some of the protamines have been extracted, thereby exposing the nuclear matrix to ATAB. This may allow the ATAB to solubilize or denature some structural nuclear matrix proteins. This possibility is supported by the demonstration that the sperm nuclei show a slight decondensation when treated with ATAB in the presence of DTT (Fig. 2). The second possibility is that there are some nuclear matrix proteins that are temporarily denatured in the presence of ATAB, exposing intermolecular cross-links necessary for the overall stability of the nuclear matrix. This may also explain why only 30% of the sperm nuclei treated with week-old NIM-ATAB/DTT were capable of participating in embryogenesis.

These data suggest that the sperm nucleus is the only component of the spermatozoa that is required for full participation in embryogenesis. They also provide the first evidence that the structural organization of DNA in the sperm nucleus may be crucial for proper development.

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