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Protein 14-3-3 eta is essential for normal meiotic spindle assembly in mouse eggs

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Abstract

The 14-3-3 proteins constitute a family of conserved, acidic proteins in a variety of organisms, regulating important intracellular events including cell cycle control, apoptosis, signal transduction and embryonic development. However, little is known about the functions of 14-3-3 in mammalian reproduction. There are seven mammalian isoforms of 14-3-3 (beta, gamma, epsilon, eta, zeta, tau/theta and sigma) encoded by different genes. We previously reported accumulation and co-localization of a specific isoform of 14-3-3, namely 14-3-3 eta, in the region of meiotic spindle apparatus in mouse eggs. To determine the role of 14-3-3 eta, we microinjected mouse oocytes with a translation-blocking morpholino oligonucleotide to knock down the expression of 14-3-3 eta mRNA. Overnight incubation of the morpholino-injected oocytes followed by *in vitro* maturation revealed absence or impairment of normal meiotic spindle assembly in eggs, as shown by immunocytochemical staining of 14-3-3 eta and alpha-tubulin along with observation of chromosomes. Duolink In Situ Proximity Ligation Assay (PLA) indicated marked interaction of 14-3-3 eta with alpha-tubulin in the region of meiotic spindle in all eggs examined, with prominent cortical accumulation about the spindles. These results suggest that 14-3-3 eta is necessary for normal meiotic spindle formation in mouse eggs. The study will help to elucidate the functional importance of 14-3-3 proteins in regulating mammalian oocyte maturation and female reproductive development.

Background and Significance

In female mammals, meiosis is initiated prenatally and oocytes remain arrested in an immature state at late prophase of the first meiotic division for long. This arrest is released as a result of the pre-ovulatory surge in luteinizing hormone – oocytes resume and complete first meiotic division and arrest at metaphase II of meiosis to form the mature, fertilizable egg. 14-3-3 is a family of conserved, homologous proteins encoded by separate genes, with a monomeric molecular mass of approximately 30 kDa, which interact with many other proteins and regulate various important cellular processes (1). To examine the role of 14-3-3 in oocyte maturation we previously identified all seven mammalian isoforms of 14-3-3 in mouse ovaries, oocytes and eggs, and showed that 14-3-3 eta accumulates and co-localizes in the region of meiotic spindle in eggs (2). Morpholino oligomers are small, ~25 base sequences of synthetic nucleotides comprising standard nucleic acid bases attached to morpholine rings linked by phosphorodiamidate groups (Figure 1a). They are an antisense technology to knock down expression of genes by blocking translation or normal splicing of mRNAs (3). Translation-blocking morpholinos bind to 5'-UTR of target mRNA and prevent protein synthesis by inhibiting ribosomal initiation complex progression (Figure 1b). To examine a possible role of 14-3-3 eta in meiotic spindle formation, we microinjected mouse oocytes with a translation-blocking morpholino oligonucleotide against 14-3-3 eta. To determine if 14-3-3 eta interacts with tubulin (a major component of spindle microtubules) in eggs, we performed the Duolink In Situ Proximity Ligation Assay – a relatively new and advanced technique that enables detection and visualization of the actual intracellular sites of the protein-protein interactions in cells. These results will enable a better understanding of the functional significance of 14-3-3 proteins in regulation of mouse oocyte maturation. The role of 14-3-3 eta in mammalian oogenesis may help unravel one of the molecular bases of female infertility, and may form a target for development of a female contraceptive.

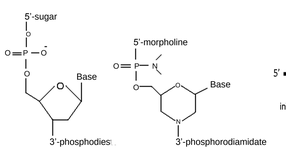

Figure 1a. Chemical structure of morpholino oligonucleotide (right) in comparison with that of DNA oligonucleotide (left).

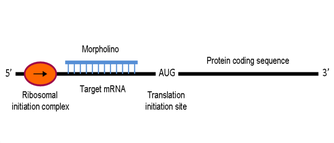

Figure 1b. Mechanism of action of translation-blocking morpholino oligo by binding to 5'-UTR of target mRNA and blocking ribosomal initiation complex progression from 5'-cap to the translation start codon.

Methods

1. a) Collection of oocytes: Immature, GV-intact oocytes were isolated from adult CF1 mice primed with eCG following standard procedure, in Hepes-buffered Minimal Essential Medium (MEM) with dibutyryl cAMP to prevent spontaneous maturation.

b) Microinjections: 10-14 pL of a 2 mM stock translation-blocking morpholino oligo (5'-CTGCTCTCGATCCCCCATGTCGCTC-3') (GeneTools, LLC) against mouse 14-3-3 eta mRNA were microinjected into cytoplasm of each oocyte (Figure 2). Controls included oocytes injected with identical volume of deionised water or a non-sense morpholino (2 mM stock invert of morpholino against 14-3-3 eta), along with some uninjected oocytes. The cells were incubated for 24 hours in bicarbonate-buffered MEM drop cultures (in humidified chamber with 5% CO2 at 37˚ C) and allowed to mature into eggs overnight in media without dibutyryl cAMP.

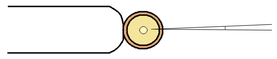

Figure 2. Schematic diagram showing intracytoplasmic microinjection of a solution taken in an injection pipette (right) into a mouse oocyte (center) held by a holding pipette (left).

c) Immunocytochemistry and Confocal Microscopy: Cells were processed for immunofluorescence staining of 14-3-3 eta and tubulin by standard procedure, using rabbit anti 14-3-3 eta (AbD Serotec) and rat anti alpha-tubulin (Santa Cruz) as primary antibodies, and 549-conjugated donkey anti rabbit and FITC-conjugated goat anti rat (Jackson Labs) as secondary antibodies. Chromosomes were stained with Hoechst dye. Cells were imaged with Olympus Fluoview FV1000 confocal microscopy system (60X oil immersion lens and various confocal zooms) at multiple confocal planes.

2. Duolink In Situ Proximity Ligation Assay (PLA): Cumulus-free eggs were isolated from adult CF1 mice by superovulation by standard procedure and processed as for immunocytochemistry, using PLA kit reagents (Olink Bioscience) by manufacturer's protocol (Figure 4). Primary antibodies used were rabbit anti 14-3-3 eta (AbD Serotec) and goat anti alpha-tubulin (ARP).

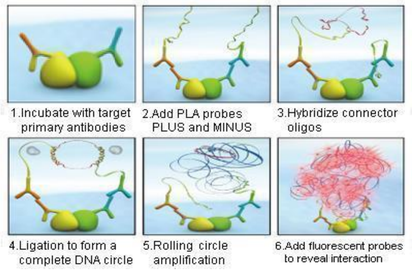

Figure 3. Schematic outline of Duolink In Situ Proximity Ligation Assay (figure courtesy: Olink Bioscience).

Results

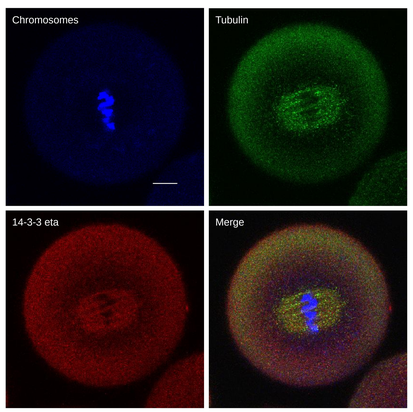

Figure 4. Representative uninjected control egg showing accumulation and co-localization of 14-3-3 eta at normal bipolar meiotic spindle, as observed by immunofluorescence staining of tubulin (green), 14-3-3 eta (red) and chromosomes (blue) along with merged image. Scale bar represents 10 µm.

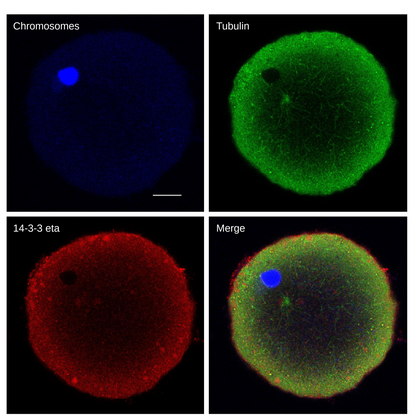

Figure 5. Representative egg microinjected with morpholino against 14-3-3 eta, showing absence of meiotic spindle assembly, as observed by immunofluorescence staining of tubulin (green), 14-3-3 eta (red) and chromosomes (blue) along with merged image. Scale bar represents 10 µm.

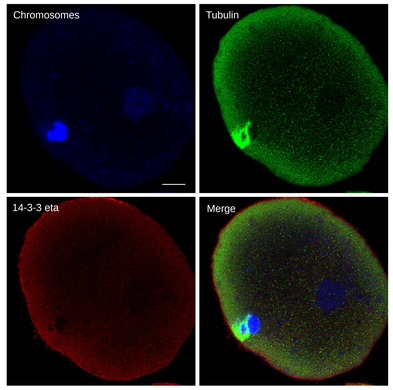

Figure 6. Representative egg microinjected with morpholino against 14-3-3 eta, showing deformed meiotic spindle assembly as observed by immunofluorescence staining of tubulin (green), 14-3-3 eta (red) and chromosomes (blue) along with merged image. Scale bar represents 10 µm.

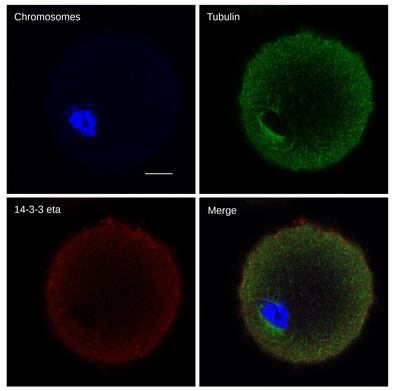

Figure 7. Representative egg microinjected with morpholino against 14-3-3 eta, showing impaired meiotic spindle assembly as observed by immunofluorescence staining of tubulin (green), 14-3-3 eta (red) and chromosomes (blue) along with merged image. Scale bar represents 10 µm.

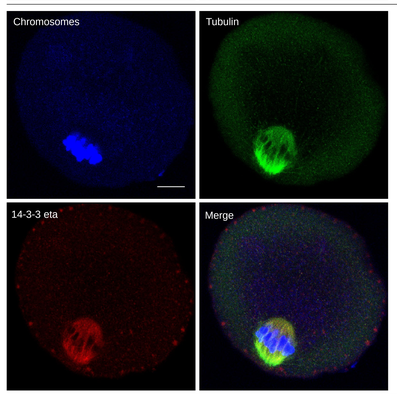

Figure 8. Representative control egg microinjected with invert morpholino against 14-3-3 eta, showing normal meiotic spindle assembly as observed by immunofluorescence staining of tubulin (green), 14-3-3 eta (red) and chromosomes (blue) along with merged image. Scale bar represents 10 µm.

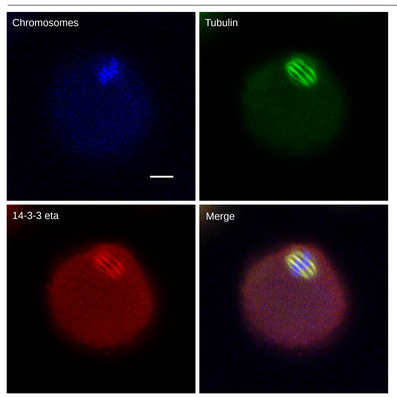

Figure 9. Representative control egg microinjected with deionised water, showing normal meiotic spindle assembly as observed by immunofluorescence staining of tubulin (green), 14-3-3 eta (red) and chromosomes (blue) along with merged image. Scale bar represents 10 µm.

Spindle assembly	14-3-3 eta morpholino	Invert 14-3-3 eta morpholino	Deionised water	Uninjected
Absent	12/41 (29.3%)	0/8 (0%)	0/10 (0%)	0/12 (0%)
Deformed	20/41 (48.8%)	1/8 (12.5%)	0/10 (0%)	0/12 (0%)
Normal	9/41 (21.9%)	7/8 (87.5%)	10/10 (100%)	12/12 (100%)

Table 1. Eggs with normal, deformed or absent spindle assembly at different microinjection conditions, out of total number of eggs observed in 5 experiments.

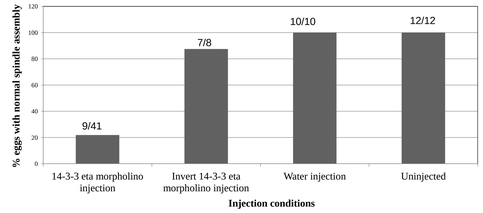

Figure 10. Eggs (%) with normal meiotic spindle assembly after microinjection of 14-3-3 eta morpholino, invert 14-3-3 eta morpholino, deionised water or no injection.

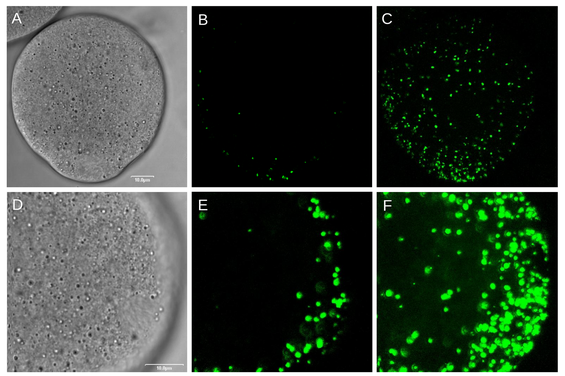

Figure 11. Representative confocal microscopy images showing Duolink In Situ PLA sites of interaction of 14-3-3 eta with tubulin in mouse eggs. The bright fluorescent Duolink reaction spots reveal the actual protein-protein binding sites. A-C indicate the same whole egg cell (A: brightfield image; B-C: fluorescence images); D-F indicate a higher magnification image of meiotic spindle in a different egg (D: brightfield image; E-F: fluorescence images). A, B, D and E display images captured after single scan through the cells. C and F show compressed z-stack images to display the interaction sites throughout the cells. Each scale bar represents 10 µm. No Duolink reaction spot was observed in control eggs treated simultaneously in absence of primary antibodies (not shown here).

Conclusions

- We have shown, for the first time, the functional importance of a specific isoform of 14-3-3, namely 14-3-3 eta, in regulating meiotic spindle assembly in mouse eggs, by morpholino-mediated antisense knock-down of the protein.
- We also detected marked interaction between 14-3-3 eta and alpha-tubulin, co-localized in the region of meiotic spindle in mouse eggs, along with distinct cortical accumulation of the interactions about the spindles. This suggests the possibility that 14-3-3 eta regulates formation and controls the organization of meiotic spindle microtubules by interacting with tubulin and/or other protein(s).
- These results will thus help to elucidate the role(s) of 14-3-3 proteins in mammalian oogenesis, oocyte maturation and female reproductive development.

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