Clathrin is essential for meiotic spindle function in oocytes

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Abstract

In the mammalian ovary, oocytes are arrested at prophase of meiosis I until a hormonal stimulus triggers resumption of meiosis. During the subsequent meiotic maturation process, which includes completion of the first meiotic division and formation of the second metaphase spindle, oocytes acquire competence for fertilization. Recently, it was shown that clathrin, a cytosolic protein complex originally defined for its role in intracellular membrane traffic, is also involved in the stabilization of kinetochore fibers in mitotic spindles of dividing somatic cells. However, whether clathrin has a similar function in meiotic spindles in oocytes has not been investigated previously. Our results show that endogenous clathrin associates with the meiotic spindles in oocytes. To study the function of clathrin during meiotic maturation, we microinjected green fluorescent protein-tagged C-terminal and N-terminal dominant-negative clathrin protein constructs into isolated porcine oocytes prior to in vitro maturation. Both protein constructs associated with meiotic spindles similar to endogenous clathrin, but induced misalignment and clumping of chromosomes, occurrence of cytoplasmic chromatin and failure of polar body extrusion. These data demonstrate that clathrin plays a crucial role in meiotic spindle function in maturing oocytes, possibly through spindle stabilization.


Introduction

During development, mammalian oocytes are arrested at prophase I, and resume meiosis when maturation is initiated in response to the luteinizing hormone surge. Oocyte maturation is characterized by several key events, including disintegration of the nuclear envelope, also referred to as germinal vesicle breakdown (GVBD), formation of the first metaphase spindle, extrusion of the first polar body, and formation of the second metaphase spindle. In most mammals, oocytes enter a second period of meiotic arrest at metaphase II (MII), which is maintained until fertilization (Mehlmann 2005b, Richard 2007). Nondisjunction of chromosomes during the meiotic divisions of oocytes is a major source of genetic disorders in humans (Martin 2008), and it is thus important to understand the machinery that controls proper chromosome segregation. In mitotic cells, the kinetochore fibers of the metaphase spindle are associated with and stabilized by a large cytosolic protein, clathrin (Royle et al. 2005, Royle & Lagnado 2006).

Clathrin is a ubiquitously expressed protein, and has a prominent role in intracellular membrane transport pathways that rely on clathrin-coated vesicles (Kirchhausen 2000, Okamoto et al. 2000, Royle et al. 2005, Ungewickell & Hinrichsen 2007). Clathrin forms a large three-legged hexameric protein complex called triskelion, which is composed of three heterodimers of a ~190 kDa heavy chain (CHC) and a ~25 kDa light chain (CLC). Triskelions can polymerize into a polyhedral coat that drives the formation of clathrin-coated vesicles, which bud off from several membranes, including the plasma membrane and the trans-Golgi network (Kirchhausen 2000, Ungewickell & Hinrichsen 2007). A less well-known function of clathrin, independently of its role in membrane transport, is to prevent misalignment and mis-segregation of chromosomes in mitotic cells, presumably by bridging microtubules (Royle et al. 2005, Royle & Lagnado 2006). Clathrin-depleted somatic cells show diverse mitotic defects, including the activation of the spindle checkpoint and disruption of cytokinesis, culminating in aberrant chromosome segregation and incomplete cell division. Although clathrin has also been observed to associate with the second meiotic spindle in mouse oocytes (Maro et al. 1985), a role for clathrin in spindle function in maturing oocytes has not been established. Mammalian oocytes form spindles that are generally much larger than mitotic spindles, and arrest at MII after in vitro maturation, thus providing ideal conditions to study the spindle function of clathrin.

In the present study, we have investigated the role of clathrin in the meiotic progression of maturing porcine oocytes. Clathrin function at metaphase spindles
requires both the N- and C-terminal domains of CHC in somatic cells (Royle et al. 2005, Royle & Lagnado 2006, Yamauchi et al. 2008). To interfere with clathrin function, we microinjected green fluorescent protein (GFP)-tagged constructs that constituted either the N- or the C-terminal domain of porcine CHC. Our data provide the first insight into the involvement of clathrin in mammalian oocyte maturation and demonstrate that clathrin is essential for meiotic spindle function.

Results

Distribution of endogenous clathrin during oocyte maturation

To investigate the distribution of endogenous clathrin, porcine oocytes were fixed either directly after isolation or after 44 h of in vitro maturation, immunolabeled for heavy chain clathrin and analysed using confocal laser scanning microscopy. All freshly isolated oocytes were at the GV stage, whereas nearly all 44 h matured oocytes reached the MII stage. In both immature GV and MII stage oocytes, CHC was distributed throughout the cytoplasm, but predominantly in association with punctate structures at the oocyte cortex, possibly corresponding to clathrin-coated vesicles (Fig. 1A and C). In MII stage oocytes, CHC was also observed at the metaphase spindle and in the polar body (Fig. 1C). Oocytes labeled for CLC showed similar patterns for both stages (data not shown). Control oocytes labeled with identical concentrations of negative control mouse IgG showed no staining at identical confocal settings (Fig. 1B and D), demonstrating the specificity of clathrin labeling. Notably, staining for CHC was relatively low in the surrounding cumulus cells, both at the onset of maturation and at MII arrest (Fig. 1A and C). The presence of clathrin on microtubules at the meiotic spindle was confirmed by immuno-double labeling 44 h matured oocytes for CHC and tubulin (Fig. 1E–H). To confirm that CHC associated with spindle fibers, 43 h matured oocytes were exposed to 10 μM nocodazole for 1 h to depolymerize the microtubules.
that make up the spindle. Nocodazole-treated oocytes lacked both tubulin and clathrin labeling around the DNA in oocytes that showed an extruded polar body, whereas these labels were present around the DNA in DMSO-treated controls and nocodazole-treated oocytes that were allowed to recover for 1 h (Supplementary Figure 1, see section on supplementary data given at the end of this article). Collectively, these data demonstrate that the association of clathrin with the spindle relies on the presence of intact microtubules.

**Clathrin constructs**

To study clathrin function during oocyte maturation, truncated dominant-negative GFP-tagged CHC protein constructs were first generated using transfected HEK293E cells and isolated to homogeneity (see Materials and Methods section and Fig. 2A). The C-terminal construct (GFP-C-CHC) contained the CHC trimerization domain, the CLC-binding domain, and GFP- and His-tags at its N-terminus. A similar construct was previously shown to trimerize with endogenous clathrin and inhibit clathrin-mediated endocytosis in HeLa cells (Liu et al. 1998). Since trimerization of clathrin is also important for its function on spindles in somatic cells (Royle & Lagnado 2006), GFP-C-CHC was expected to interfere with the spindle function of clathrin in oocytes. The N-terminal construct (N-CHC-GFP) encompassed a domain that was previously demonstrated to interfere with clathrin-mediated endocytosis and to associate with mitotic spindles in somatic cells (Royle et al. 2005), and contained GFP- and His-tags at its C-terminus. We also tried to produce these dominant-negative CHC protein constructs with GFP-tags on the other side and a GFP-tagged full-length CHC construct. Unfortunately, these constructs were not stably expressed by our producer cell line. Instead, non-conjugated GFP was used as a negative control.

Isolated constructs were then microinjected into immature oocytes. To control for the amount of injected protein, total GFP fluorescence in oocytes was measured by epifluorescence microscopy (Lee 1989) directly after injection (Fig. 2B). Of successfully injected oocytes, total fluorescence did not differ significantly between oocytes injected with GFP or either one of the CHC constructs (Fig. 2C). After injection, oocytes were allowed to mature for 44 h, fixed, immunolabeled for tubulin, and stained for DNA. Consistent with the distribution of endogenous clathrin, both N-CHC-GFP and GFP-C-CHC accumulated at the meiotic spindle (Fig. 3A and B). To quantify recruitment of CHC constructs to the meiotic spindles (Royle et al. 2005), we determined GFP fluorescence intensities in representative areas on the spindle relative to the oocyte cortex (Fig. 3A–C). Both N-CHC-GFP and GFP-C-CHC were significantly enriched on the spindle in comparison with non-conjugated GFP (Fig. 3D).

**Clathrin constructs disrupt meiotic divisions in oocytes**

Our next step was to investigate the effects of injected N-CHC-GFP and GFP-C-CHC on oocyte maturation. A normal MII pattern was observed in the majority of oocytes that were injected with non-conjugated GFP.
Severe defects were observed, however, in both N-CHC-GFP- and GFP-C-CHC-injected oocytes. Both CHC constructs caused misalignment of chromosomes on the metaphase plate and formation of large clumps of condensed chromatin interspersed with patches of less condensed chromatin (Fig. 4 E–T). In addition, chromosomes were regularly observed outside the metaphase spindle (e.g. open arrowhead in Fig. 4 Q–T), and the polar body often remained connected to the oocyte via a ‘DNA bridge’ (open arrowheads in Fig. 4E–P) that varied in thickness and length between the injected oocytes (short and thin in Fig. 4E–H, short and thick in Fig. 4I–L, and long and thin in Fig. 4M–P). These bridges displayed no tubulin labeling, indicating that the spindle midbody was no longer present. These phenotypes are illustrated more clearly in Supplementary Movies 1–4 (see section on supplementary data given at the end of this article) which show 360° rotations of 3D reconstructions of the same oocytes as in Fig. 4A–H. In a subset of GFP-C-CHC-injected oocytes, condensed chromosomes were scattered throughout the area around the metaphase spindle. These scattered chromosomes formed small clusters that also contained tubulin (open arrowheads in Fig. 4Q–T) creating small spindle-like structures. Since defects at MII may result from abnormal progression through meiosis I (MI), oocytes that were arrested at MI after 44 h of maturation were also examined in detail. Typical aberrant MI morphology observed in these construct-injected oocytes included incomplete condensation and clumping of chromosomes (Fig. 6), abnormally dense astral microtubule networks (Fig. 6E–H), loose chromosomes outside the metaphase plate (filled arrowheads in Fig. 6), and tubulin fibers that connected the spindle to the loose chromosomes (open arrowheads in Fig. 6l–P).

To quantify the effects of the CHC constructs on oocyte maturation, 44 h matured oocytes were scored into four categories based on their tubulin and DNA staining patterns: aberrant GV (clearly visible nucleus), aberrant MI (with oocytes from prometaphase I up to and including telophase I), aberrant MII (clearly visible metaphase spindle and polar body, showing abnormal structure), and normal MII (clearly visible metaphase spindle and polar body). Oocytes that showed a normal GV or MI pattern after maturation were also scored as aberrant, since these oocytes failed to complete

(Figs 4A–D and 5A and B). Severe defects were observed, however, in both N-CHC-GFP- and GFP-C-CHC-injected oocytes. Both CHC constructs caused misalignment of chromosomes on the metaphase plate and formation of large clumps of condensed chromatin interspersed with patches of less condensed chromatin (Fig. 4E–T). In addition, chromosomes were regularly observed outside the metaphase spindle (e.g. open arrowhead in Fig. 4 Q–T), and the polar body often remained connected to the oocyte via a ‘DNA bridge’ (open arrowheads in Fig. 4E–P) that varied in thickness and length between the injected oocytes (short and thin in Fig. 4E–H, short and thick in Fig. 4I–L, and long and thin in Fig. 4M–P). These bridges displayed no tubulin labeling, indicating that the spindle midbody was no longer present. These phenotypes are illustrated more clearly in Supplementary Movies 1–4 (see section on supplementary data given at the end of this article) which show 360° rotations of 3D reconstructions of the same oocytes as in Fig. 4A–H. In a subset of GFP-C-CHC-injected oocytes, condensed chromosomes were scattered throughout the area around the metaphase spindle. These scattered chromosomes formed small clusters that also contained tubulin (open arrowheads in Fig. 4Q–T) creating small spindle-like structures. Since defects at MII may result from abnormal progression through meiosis I (MI), oocytes that were arrested at MI after 44 h of maturation were also examined in detail. Typical aberrant MI morphology observed in these construct-injected oocytes included incomplete condensation and clumping of chromosomes (Fig. 6), abnormally dense astral microtubule networks (Fig. 6E–H), loose chromosomes outside the metaphase plate (filled arrowheads in Fig. 6), and tubulin fibers that connected the spindle to the loose chromosomes (open arrowheads in Fig. 6l–P).

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maturation. Oocytes that could not be scored into any of these categories (~4%) were excluded from the analysis. Of the non-injected and GFP-injected oocytes, 60–70% showed normal morphology at MII (Fig. 5A and B). In contrast, of the N-CHC-GFP- and GFP-C-CHC-injected oocytes, only 13 and 2% respectively reached normal MII, while most showed an aberrant MII morphology. Statistical analysis of these data shows that oocytes injected with N-CHC-GFP or GFP-C-CHC were significantly inhibited in progressing through maturation as compared with GFP-injected oocytes. In both N-CHC-GFP and GFP-C-CHC conditions, the strongest significant effect was found for the ratio of aberrant versus normal MII oocytes compared with that in the control group. The ratio of aberrant MI versus normal MII oocytes was, however, also significantly higher for both CHC constructs. Only GFP-C-CHC-injected oocytes differed significantly in comparison with the control group in the ratio of aberrant GV versus normal MII phenotypes. A summary of these results and their significance is shown in Fig. 5C.

To specify MII aberrancies in more detail, we scored aberrant MII oocytes by phenotype using the following categories: misaligned chromosomes (chromatin outside the equator of the MII spindle), chromosome clumps (large clumps of condensed chromatin interspersed with patches of less condensed chromatin), DNA bridge (direct link between chromatin in the polar body and the oocyte) and cytoplasmic chromatin (loose chromosomes present in the cytoplasm). Almost all oocytes injected with either one of the CHC constructs showed misaligned chromosomes and chromosome clumps, whereas DNA bridges and cytoplasmic chromatin were observed in only 20–45% of oocytes. Statistical analysis of these results using $\chi^2$ tests did not reveal any significant differences between N-CHC-GFP- and GFP-C-CHC-injected oocytes, suggesting that the effects of these constructs on oocyte maturation were identical (Fig. 5D).
Figure 5 Quantification of aberrant meiotic profiles after injection of clathrin constructs. (A and B) Bar graphs showing percentages of 44 h matured oocytes at various stages after injection as determined in Fig. 4. Bars represent weighted mean percentage ± weighted s.e.m. (N=3 independent experiments) of oocytes at the indicated stages. The indicated conditions are non-injected control oocytes (NIC; 67 and 62 oocytes in A and B respectively), GFP-injected oocytes (GFP; 58 and 50 oocytes in A and B respectively), N-CHC-GFP-injected oocytes (N-CHC-GFP; A; 61 oocytes), and GFP-C-CHC-injected oocytes (GFP-C-CHC; B; 58 oocytes). Significance of the results is indicated in the results section. (C) Table showing a summary of the analysis of the data shown in (A) and (B). The ratio of oocytes at each aberrant stage versus oocytes at the reference stage (normal MII) was compared between NIC/N-CHC-GFP/GFP-C-CHC and GFP conditions using multinomial logistic regression models. Several models were employed to investigate the interaction between maturation stage, which was used as an ordinal variable, and condition. Based on a minimized Akaike information criterion (AIC) value, a model that incorporated both N-CHC-GFP and GFP-C-CHC conditions and their respective controls, and used experiment number to control for random effects was chosen for final analysis. Significance of the results is indicated by asterisks (*P < 0.05; ** P < 0.01).

(D) Aberrant MII oocytes in (A) and (B) were scored for observed defects and analyzed by χ² tests, which did not reveal any significant differences between N-CHC-GFP- and GFP-C-CHC-injected oocytes (P>0.05; Bonferroni corrected).

Discussion

In this study, we have demonstrated that clathrin is associated with meiotic spindles in porcine oocytes, and that interference with clathrin function by injection of dominant-negative CHC protein constructs disrupted meiosis progression. Moreover, our results support the notion that clathrin is involved in chromosome segregation during oocyte meiosis.

Clathrin is a cytosolic protein that, in addition to its role in membrane transport, was recently shown to stabilize the mitotic spindle in somatic cells (Royle et al. 2005, Royle & Lagnado 2006). This role for clathrin in mitosis was later questioned for a chicken pre-B lymphoma cell line (DKO-R). Although the localization of clathrin at the spindle during mitosis is highly consistent between different cell lines, clathrin-depleted DKO-R cells did not show mitotic defects (Royle et al. 2005, Borlido et al. 2008). These data suggest the existence of cell type-specific differences in spindle-associated clathrin function. It should be noted, however, that DKO-R cells were specifically developed to study clathrin function while preventing apoptosis caused by clathrin depletion, and could have acquired adaptations that attenuate the mitotic phenotype of clathrin depletion that was observed in other cell lines (Wettey et al. 2002, Royle et al. 2005, Borlido et al. 2008). Although the necessity of clathrin function on mitotic spindles may vary between different cell lines, a conserved role for clathrin in stabilizing meiotic spindles is plausible, given that spindle instability is closely linked to aneuploidy (Jones 2008). Although clathrin also serves as an important factor in intracellular membrane transport (Kirchhausen 2000, Ungewickell & Hinrichsen 2007), the potential functions of clathrin in oocytes have not been investigated previously. Consistent with previous observations for mouse MII oocytes (Maro et al. 1985), we demonstrate by immunofluorescence microscopy that clathrin associates with metaphase spindles in porcine oocytes (Fig. 1). The initial observations by Maro et al. (1985) were thought to reflect association of clathrin-coated vesicles with spindle fibers. In somatic cells, however, clathrin was shown to bind the mitotic spindle directly, while membranes did...
not associate with the spindle (Royle et al. 2005, Royle & Lagnado 2006). Using lipophilic dyes, we could not detect association of membranes with the meiotic spindle in oocytes (data not shown). Moreover, clathrin did not persist at the spindle upon depolymerization of spindle microtubules using nocodazole, and was recovered together with spindle microtubules within 1 h of nocodazole removal (Supplementary Figure 1). These data suggest that clathrin does not associate with the spindle matrix, as nocodazole-mediated depolymerization of the spindle in mitotic cells was previously shown to leave the spindle matrix in place (Lince-Faria et al. 2009).

Unlike somatic cells, fully grown mammalian oocytes are characterized by repressed transcription (Motlik et al. 1984, Fair et al. 1996, Bouniol-Baly et al. 1999, De La Fuente & Eppig 2001, Bjerregaard & Maddox-Hyttel 2004), and oocytes thus cannot be manipulated by cDNA transfection. Given the ubiquitous and crucial functions of clathrin, clathrin knockout animals have not been generated, and conditional knockout systems for clathrin in oocytes are not available. RNAi knockdown of endogenous clathrin in oocytes is also extremely unlikely to succeed, because clathrin is one of the most abundant proteins in cells (Goud et al. 1985), and has a very long half-life (Acton & Brodsky 1990). Indeed, even in highly active mitotic cells, CHC RNAi does not show an effect on the mitotic index until 48 h after transfection (Royle et al. 2005). Although exogenous proteins can be expressed by injection of mRNA in porcine oocytes, the efficiency of such injection procedures is low (~30% of injected oocytes showed expression during maturation), and expression levels vary extensively between oocytes (Ohashi et al. 2001). Instead, the method used here to microinject dominant-negative clathrin protein constructs was highly efficient (~75% of injected oocytes showed sustained fluorescence), and allowed instantaneous introduction of a reproducible amount of exogenous protein into each oocyte (Fig. 2). These microinjected GFP-tagged constructs, corresponding to the N- or C-terminal domains of CHC (Fig. 2), were both recruited to MII spindles (Fig. 3). N-CHC-GFP, which contains the previously determined spindle-binding domain (Royle et al. 2005), was recruited to the meiotic spindle to the same extent as GFP-C-CHC (Fig. 3). These observations suggest that both constructs contain spindle recruitment domains, although it cannot be excluded that our constructs have been recruited to the spindle by associated endogenous full-length CHC that may have been incorporated in ‘mixed’ triskelions. A recent study showed that formation of a complex, which consists of clathrin, B-Myb, and filamin, is required for recruitment of clathrin to the mitotic spindle in somatic cells, and the authors suggested that the C-terminal trimerization...
domain of clathrin could also be involved in the formation of this complex (Yamauchi et al. 2008). In another study, the N-terminal domain appeared to be indispensable for spindle localization, since a construct lacking this domain (331–1639) failed to be recruited to mitotic spindles (Royle & Lagano 2006). It should be noted that these results were obtained in somatic cells that had been depleted of endogenous clathrin by RNAi, and as such did not allow formation of mixed triskelions containing both endogenous CHC and the truncated CHC construct.

Oocytes injected with either one of our CHC constructs displayed severe defects in MI morphology (Figs 4A and 5) while maturation rates and MI morphology of GFP-injected control oocytes did not differ from those of non-injected controls (Figs 4A–D and 5A and B). Both CHC constructs caused misalignment of chromosomes and formation of chromosome clumps, which may be explained by CHC construct-induced failure of the spindle checkpoint during MI. When some homologous chromosomes separate normally and others remain attached to each other and are pulled either into the nascent polar body or into the oocyte, chromosome clumps would form on the second metaphase spindle. Moreover, the aberrant presence of homologous chromosome pairs on the second metaphase spindle may disrupt proper chromosome alignment. Indeed, oocytes that were arrested at MI after 44 h of maturation as a consequence of the injection of CHC constructs showed congression failure (Fig. 6). In spite of this severe phenotype observed in some of the injected oocytes, the majority of oocytes that were injected with the CHC constructs did not arrest at MI (Fig. 5A and B), indicating failure of the MI spindle checkpoint. Although these inferences remain speculative, it is clear that future research on clathrin function in meiotic spindles should include investigation of checkpoint controls, particularly since clathrin adaptor proteins have been implicated in spindle function and checkpoint control in mitotic cells (Cayrol et al. 2002, Lehtonen et al. 2008).

Clathrin-mediated endocytosis may also have been affected by our CHC constructs. A possible effect of interference with endocytosis is failure of polar body cytokinesis, since it has been demonstrated that endocytosis at the cleavage furrow is important for cytokinesis in several types of mitotic cells (Niswonger & O’Halloran 1997, Feng et al. 2002, Schweitzer et al. 2005, Albertson et al. 2005, Warner et al. 2006, Montagnac et al. 2008). These findings suggest that endocytosis may also be important for cytokinesis in oocytes. We observed that respectively 44 and 19% of N-CHC-GFP- or GFP-C-CHC-injected oocytes remained connected to their polar body via a DNA bridge (Figs 4E–P and 5D), demonstrating that chromosome segregation is incomplete in these oocytes. These cytoplasmic connections between oocytes and their polar bodies did not contain tubulin, suggesting that the spindle midbody, which may be involved in abscission during cytokinesis (Montagnac & Chavrier 2008), was no longer present. These results are indicative of destabilization of the first meiotic spindle, given that cytokinesis is normally preceded by chromosome segregation. We conclude that interference with endocytosis-dependent cytokinesis cannot explain our results. Studies on Xenopus oocytes demonstrated that meiosis resumption involves endocytosis of a G-protein-coupled receptor (Wang & Liu 2003, El-Jouni et al. 2007). The G-protein-coupled receptor GPR3 appears to be required for maintenance of meiotic arrest in mouse, rat, and human oocytes (Mehlmann et al. 2004, Hinckley et al. 2005, Mehlmann 2005a, DiLuigi et al. 2008), suggesting that endocytosis of this receptor may also be involved in resumption of oocyte meiosis in mammals. Since clathrin is required for endocytosis of specific receptors in somatic cells (Kirchhausen 2000, Moore et al. 2007, Ungewickell & Hinrichsen 2007), these findings suggest an alternative role for clathrin in resumption of meiosis. Constructs similar to our CHC constructs have been demonstrated to interfere with membrane traffic in somatic cells (Liu et al. 1998, Royle et al. 2005). In our study, however, meiosis progression beyond GVBD was not affected in the majority of CHC construct-injected oocytes (Fig. 5A and B). Therefore, inhibition of G-protein-coupled receptor endocytosis cannot explain our results.

Taken together, our findings in porcine oocytes are consistent with a role for clathrin in stabilizing metaphase spindles (Royle et al. 2005, Royle & Lagano 2006). Disruption of clathrin function in maturing oocytes causes severe spindle-related defects, which may be due to spindle checkpoint failure and/or spindle destabilization during MI. Although we have established that clathrin plays a pivotal role in meiosis progression during porcine oocyte maturation, further research will be required to untangle the contributions of its functions to meiotic spindle stability.

Materials and Methods

Recombinant clathrin constructs

Porcine clathrin heavy chain was PCR amplified from porcine brain cDNA using the Expand Long Template PCR System (ELT-PCR; Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s instructions with primers designed based on homology between the known human (NM_004859), bovine (NM_174023), and mouse (NM_001003908) CHC sequences (Table 1, Primers for PCR), and sequenced (Baseclear, Leiden, The Netherlands; EMBL accession number FM210346, accession date 3 March 2009). The PCR product was then diluted 1:1000 and amplified again using Phu DNA polymerase (Fermentas, Burlington, Ontario, Canada) to obtain coding cDNA for residues 1–479 (N-CHC) and to introduce in-frame residues 1073–1675 (C-CHC) and to introduce in-frame BamH I – NotI restriction sites for C-terminal clathrin and
Full-length clathrin 5

Cloning primers

<table>
<thead>
<tr>
<th>Primers for PCR</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Full-length clathrin</td>
<td>5'-GGGA GGA CAC CAT ACC CCC CGA CAG-3'</td>
<td>5'-AAC AGA TTA ATT AAG CAG T-3'</td>
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<tr>
<td>C-terminal clathrin</td>
<td>5'-GGG GTG TCG GAT CCG CCC AGA TCC TTC</td>
<td>5'-GGG GCC GCC AGT CTG TAC CCA AAG CCA</td>
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<tr>
<td>N-terminal clathrin</td>
<td>5'-GGG GCC GCC AGT TAC CCA AAG CCA</td>
<td>5'-GGG GCC GCC AGT TAC CCA AAG CCA</td>
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BsaI – NotI

Both constructs were cloned into pCR4-TOPO (Invitrogen, Paisley, UK), and positive clones were sequenced. The predicted N- and C-terminal CHC constructs were then ligated into pUPE (U-Protein Express BV, Utrecht, The Netherlands) containing the Superglo GFP sequence followed by a His-tag at the N-terminus of the C-terminal construct and at the C-terminus of the N-terminal construct. Recombinant CHC constructs were expressed in HEK293E cells. HEK293E cells from a 1-l suspension culture were pelleted by centrifugation and resuspended in 0.5 M Tris–HCl, pH 7.5 containing protease inhibitor (Roche), lysed by repeated freeze–thawing and further homogenized in a Potter-Elvehjem homogenizer. Nuclei, organelles, and large protein complexes were removed by sequential centrifugation steps at 1000 g for 20 min, 20 000 g for 20 min, and finally 200 000 g for 1.5 h.

Protein constructs were purified from the resulting supernatant by Ni2+-affinity chromatography (Ni Sepharose FF, GE Healthcare). The yields of N- and C-terminal clathrin protein constructs were subsequently subjected to gel filtration (Superdex 200 pg 16/8, GE Healthcare). The yields of N- and C-terminal clathrin heavy chain protein constructs were identical between experiments.

In vitro maturation was performed as described previously (Hölzenspies et al., 2009). OCCs were washed and denuded by gentle pipetting in 80 mM 37°C 3% (w/v) PVP, pH 6.9 at 37°C (PEM–PVP), and then fixed in PEM–PVP containing 4% (v/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) at room temperature for 1 h. After fixation, OCCs were washed twice in 0.1 M phosphate buffer (pH 7.4) containing 0.3% (w/v) PVP, and permeabilized in 0.1 M phosphate buffer supplemented with 0.1% (w/v) saponin (Riedel de Haen AG, Seelze, Germany). Permeabilized OCCs were blocked in 0.1 M phosphate buffer containing 0.1% (w/v) 0.25 mM sodium pyruvate, 10 mM sodium lactate, 0.5 mM MgCl2·6H2O, 2 mM CaCl2·2H2O, 10 mM HEPES, 0.1% (w/v) polyvinylpyrrolidone (PVP), 1 mM dibutyryl cAMP (dbcAMP), pH 7.4. Oocytes were maintained in prophase arrest during collection and microinjection by the presence of dbcAMP. Prior to injection, oocytes were partially denuded by gentle pipetting until 3–6 layers of cumulus remained, and washed in HEPES-buffered M199 from Gibco BRL (Grand Island, NY, USA) supplemented with 1 mM dbcAMP. For injection, groups of five oocytes were placed in 5 µl drops of this medium under oil at 37°C on an IX71 inverted microscope (Olympus, Zoeterwoude, The Netherlands) equipped with a heated stage and an epifluorescence setup. A suction pipette was used to immobilize oocytes, and beveled rigid borosilicate micropipettes with a 30° angle and a 3.5 µm tip diameter (custom tips; Eppendorf, Hamburg, Germany) were used to perform injections. Injection solutions were centrifuged for 10 min at 16 000 g just prior to use, and ~1 µl was backloaded into the micropipettes, which were subsequently connected to a Femtojet pressure injection system (Eppendorf). Immediately after injection, epifluorescent images were recorded on the IX71 microscope at 100× magnification through a FITC filter using a DP20 camera (Olympus) at 100 ms exposure. Any oocytes showing much higher or lower fluorescence intensity than average (typically 20–30% of injected oocytes) were immediately removed from the experiment (see for example Fig. 2B). To ensure that equal amounts had been injected between groups, the epifluorescence images taken during injection experiments were evaluated by measuring the total GFP fluorescence per oocyte in ImageJ. Total fluorescence was measured in regions of interest (ROIs) around injected oocytes. ROIs were generated using a fixed threshold, which was kept identical between experiments. In vitro maturation was performed as described previously (Hölzenspies et al., 2009).

Immunofluorescence and confocal microscopy

All chemicals were purchased from Sigma Chemical Co., unless otherwise indicated. Cumulus–oocyte complexes (COCs) were collected by aspiration from 3 to 6 mm follicles from sow (Sus scrofa) ovaries, which were acquired from a slaughterhouse. The collected COCs were allowed to settle and washed several times in a buffer containing 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO3, 0.4 mM NaH2PO4·H2O, 5 mM EGTA, 2 mM MgCl2, 0.3% (w/v) PVP, pH 6.9 at 37°C (PEM–PVP), and then fixed in PEMS–PVP containing 4% (v/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) at room temperature for 1 h. After fixation, oocytes were washed twice in 0.1 M phosphate buffer (pH 7.4) containing 0.3% (w/v) PVP, and permeabilized in 0.1 M phosphate buffer supplemented with 0.1% (w/v) saponin (Riedel de Haen AG, Seelze, Germany). Permeabilized oocytes were blocked in 0.1 M phosphate buffer containing 0.1% (w/v) www.reproduction-online.org
saponin, 1% (w/v) BSA and 2% (v/v) normal goat serum (Vector Lab, Burlingame, CA, USA) supplemented with 100 mM glycine for 2 h at room temperature. After this blocking step, oocytes were subjected to sequential 1 h incubations with primary and secondary antibodies, which had been diluted in blocking solution without glycine and centrifuged at 100,000 g for 1 h before use. To label microtubules, a mixture of rabbit polyclonal antibodies directed against α-tubulin (1:400; Abcam, Cambridge, UK) and β-tubulin (1:400; Sigma) was used. Clathrin was labeled with CON.1, which is directed against a conserved region (AA 23–44) of CLC (Nathke et al. 1992), and X22, which is directed against a conserved region (AA 1109–1128) near the C-terminus of CHC (Brodsky 1985, Liu et al. 1995). CON.1 and X22 were produced from hybridoma cultures in our lab (kindly provided by Dr F M Brodsky). Irrelevant primary antibodies of the same isotype (Sigma) and at identical concentrations were used as negative controls. Secondary antibodies were goat anti-mouse IgG alexa488 and goat anti-rabbit IgG alexa568 (Molecular Probes, Eugene, OR, USA), and DNA was labeled for 20 min with 10 μM TO-PRO-3 iodide (Molecular Probes). Each labeling step was followed by three washes in 0.1 M phosphate buffer supplemented with 0.1% (w/v) saponin. Finally, oocytes were sealed in Vectashield (Vector Lab) on Superfrost Plus microscope slides (Menzel, Braunschweig, Germany) in 0.12 mm Secure-Seal Spacers (Molecular Probes).

Confocal images were obtained using a Nikon eclipse TE300 microscope (Nikon Corp., Tokyo, Japan) equipped with a 40× oil immersion objective (N.A. 1.3) and a Bio-Rad Radiance 2100MP confocal system (Zeiss/Bio-Rad, Hertfordshire, UK). Multichannel images were recorded by sequential excitation using 488-, 543-, and 637-nm lasers. Images were acquired using settings adjusted for submaximal confocal settings (at least 50% of extracellular pixel values below threshold value and 0.1–0.5% of pixel values inside the oocyte above maximal pixel value), and analysed using ImageJ (NIH; http://rsb.info.nih.gov/ij/). Submaximal confocal settings were also used to measure spindle recruitment since variations in spindle location and orientation cause large differences in diffraction due to the large size and high lipid content of oocytes (Supplementary Movie 5, see section on supplementary data given at the end of this article). Spindle recruitment was determined by dividing GFP fluorescence intensity on meiotic spindles by that in cortical regions as indicated. Images shown are maximum intensity Z projections of six consecutive confocal sections (unless otherwise indicated) taken at 2 μm intervals and subjected to limited contrast/brightness enhancements up to ~20%. Control images were produced with the highest settings and enhancements used to create images of specific antibody stainings from the same experiment. Supplementary Movies were produced using ImageJ and the VolumeJ plugin (Abramoff & Viergever 2002) as described in the supplementary data.

Statistical analysis

Student’s t-tests and χ² tests were performed in Microsoft Excel, and multinominal regression was performed in the R environment (www.r-project.org) using the ‘lmer’ function of the lme4 package (R package version 0.999375-28, http://lme4.r-forge.r-project.org). P values were considered significant at P<0.05 (Bonferroni corrected). Mean percentages and s.e.m. were weighted by the number of oocytes in each experiment.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-10-0045.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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