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Meiotic maturation failure induced by DICER1 deficiency is derived from primary oocyte ooplasm

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Abstract

RNA interference (RNAi) has diverse functions across cellular processes, including a role in the development of the mammalian oocyte. Mouse primary oocytes deficient in the key RNAi enzyme DICER1 exhibit pronounced defects in chromosome congression and spindle formation during meiotic maturation. The cause of this meiotic maturation failure is unknown. In this study, observations of chromosomes and spindle microtubules during prometaphase in DICER1-deficient oocytes indicate that chromosome congression and spindle formation are overtly normal. Spindle breakdown and chromosome displacement occur after the metaphase plate has formed, during the metaphase to anaphase transition. We hypothesised that this defect could be attributed to either RNAi-mediated regulation of nuclear factors, such as the regulation of centromere chromatin assembly, or the regulation of mRNA expression within the cytoplasm. By transplanting germinal vesicles between DICER1-deficient and wild-type primary oocytes, we show that, unexpectedly, the meiotic failure is not caused by a deficiency derived from the germinal vesicle component. Instead, we reveal that the ooplasm of primary oocytes contains DICER1-dependent factors that are crucial for chromosome segregation and meiotic maturation.

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Introduction

The onset of meiotic maturation is marked in fully grown primary oocytes by nuclear dissolution or germinal vesicle breakdown (GVBD). Mouse primary oocytes induced to be deficient in the key RNA interference (RNAi) enzyme 'Dicer1, Dcr-1 homologue (*Drosophila*)' (DICER1), at an early stage in their growth phase are able to grow to full size, undergo GVBD and be ovulated. However, when examined at the stage when meiosis I should be complete, they have pronounced and variable meiotic defects, with most having failed in extrusion of the first polar body (PB1E; Murchison *et al*. 2007, Tang *et al*. 2007). At this point, oocytes quickly degenerate. The cause of this meiotic defect is unknown. Small RNAs such as micro RNAs (miRNAs) and short interfering RNAs (siRNAs), which are the products of DICER1 processing, have a pervading influence on biological processes, and have been implicated in transcriptional and post-transcriptional regulation of mRNA levels, along with the regulation of translation (Liu *et al*. 2004, Matzke & Birchler 2005). As meiosis is a complex and intricately regulated process, the DICER1 pathway could be influencing a number of meiotic mechanisms.

Oocytes are arrested in prophase I until recruitment into the growing phase and initiation of meiotic maturation. At ovulation, oocytes complete meiosis I, extrude a polar body and arrest at metaphase of meiosis II, awaiting fertilisation. This meiotic maturation occurs in the absence of transcription, and is entirely dependent on the translation of stores of maternal transcripts and localisation of proteins. By the end of prophase, or GVBD, fundamental structures such as the centromere and kinetochore, essential for chromosome congression and segregation, are essentially fully assembled. The assembly of both of these systems relies on the successful establishment of centric heterochromatin by DICER1-dependent RNAi mechanisms, which are thought to be universal in eukaryotes (Bernard *et al*. 2001, Choo 2001, Volpe *et al*. 2003, Fukagawa *et al*. 2004, Motamedi *et al*. 2004, Pal-Bhadra *et al*. 2004, Folco *et al*. 2008). Therefore, for DICER1-deficient

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oocytes, defects in centromere heterochromatinisation, with consequent defects in kinetochore assembly, would seem likely causes of meiotic maturation failure. Alternatively, DICER1 could play a role in the post-transcriptional control of mRNA content and expression within the oocyte cytoplasm. At what stage during development oocytes become dependent on DICER1 for any of the above processes is also unknown.

The purpose of this study was to determine at which point meiotic failure occurs in the maturation of DICER1-deficient oocytes, and to investigate whether the primary cause of meiotic failure could be attributed to either cytoplasmic or nuclear factors. Depleting DICER1 in oocytes at two different time points of oocyte development both resulted in the same meiotic failure phenotype. A time course analysis through the first meiotic division demonstrates that meiosis proceeds normally until metaphase I. Using nuclear transplantation, we have investigated the extent to which there is a deficiency in the prophase nucleus, and hence possibly in centromeric heterochromatin organisation. Results show that, rather than there being a significant deficiency residing in this component, meiotic failure in DICER1-deficient oocytes is largely derived from deficiencies in the ooplasm.

Results

Mutant *Dicer1*$^{-/-}$ oocytes generated by the *Zp3-cre* transgene exhibited the same abnormal phenotype as previously described (Murchison *et al.* 2007, Tang *et al.* 2007). *Dicer1*$^{c/-}$, *Zp3-cre*/0 females had ovaries with normal numbers of oocytes at normal stages of maturation, but were infertile (DM Mattiske, unpublished observations). Wild-type oocytes displayed bipolar metaphase spindles with chromosomes aligned along the spindle equator (Fig. 1A and B). By contrast, ovulated DICER1-deficient oocytes exhibited pronounced defects of the spindle, scattered chromosomes, PB1E failure (Fig. 1C and D), lacked detectable wild-type *Dicer1* mRNA (Fig. 2A), and were very low in miRNA content, as indicated by low expression of *Mirn16-1* and *Mirn30b* (Fig. 2B). These results confirm previous studies that demonstrate that reduced DICER1 expression results in reduced expression of siRNAs and miRNAs (Murchison *et al.* 2007, Tang *et al.* 2007, Watanabe *et al.* 2008). Interestingly, we observed this same abnormal spindle phenotype in ovulated oocytes of *Dicer1*$^{c/-}$, *Alpl-cre*/0 females (Fig. 1E and F). The only difference seen in the *Zp3-cre*- and *Alpl-cre*-mediated phenotypes was that while nearly all *Zp3-cre* derived oocytes (>90%) had abnormal spindles, approximately half of *Alpl-cre* derived oocytes had abnormal spindles – 21 out of 40 examined. In this latter set, the oocytes with normal spindles were likely to have been excision escapees, as a similar effect in oocytes was observed in previous uses of the *Alpl-cre* transgene (Kehler *et al.* 2004, Tachibana *et al.* 2007), although not as frequently as in the present study.

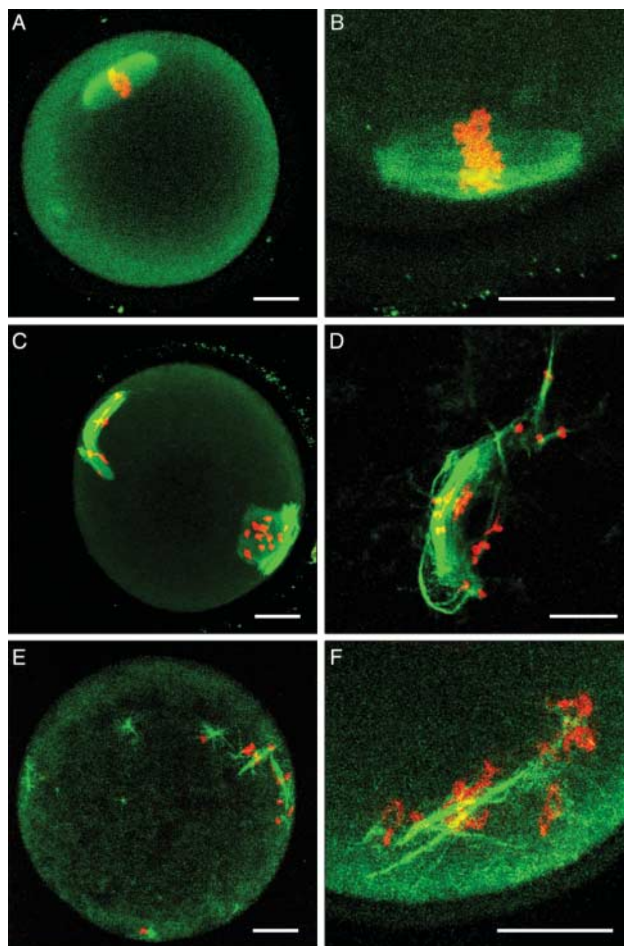

Figure 1 Phenotype of DICER1-deficient ovulated oocytes. (A and B) Wild type. (C and D) DICER1 deficient, induced by the *Zp3-cre* transgene. (E and F) Presumptive DICER1 deficient, induced by the *Alpl-cre* transgene. Green, tubulin, β-staining. Red, DNA stained with PI. Scale bars, 20 μm.

Oocytes were cultured from GV stages through to GVBD and matured for a further 8 h in order to determine when meiotic maturation failed in mutant oocytes generated by the *Zp3-cre* transgene. Two hours after GVBD, all wild-type and mutant oocytes appeared normal (Fig. 3A and E). Chromosomes had condensed and were congressing onto the forming spindle. Between 4 and 6 h after GVBD, the spindle was fully formed and chromosomes were aligning on the metaphase plate (Fig. 3B, C and F). At 6-h post GVBD, only some mutant oocytes (7 out of 23) showed signs of abnormalities (Fig. 3G). These oocytes contained a few chromosomes scattered along the metaphase spindle, rather than being aligned along the equator. By 8-h post GVBD, most wild-type oocytes (24 out of 25) expelled a polar body and formed a metaphase II spindle (Fig. 3D). However, nearly

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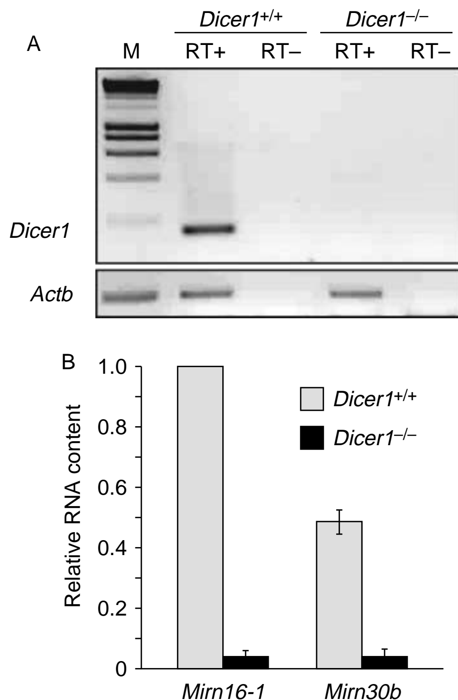

Figure 2 *Dicer1* mRNA and miRNA content in DICER1-deficient ovulated oocytes. (A) RT+, PCR amplification after incubation with reverse transcriptase. RT-, PCR amplification after incubation without reverse transcriptase. (B) The amounts of *Mirn16-1* and *Mirn30b* are relative, or normalised, to the Ct values obtained for *Actb* QPCR and are on a linear scale. Normalised Ct values were calibrated to that for *Dicer1*$^{+/+}$, *Mirn16-1*, equal to 1.0. Error bars are S.D. of values obtained in triplicate reactions.

all mutant oocytes (11 out of 12) contained spindle defects, including abnormal chromosome arrangement and multiple spindles (Fig. 3H).

GV transplantation experiments were carried out to determine the extent to which the mutant phenotype is derived from the GV or ooplasm components of the prophase oocyte, with results shown in Table 1. In these experiments, the *Zp3-cre* transgene was used for conditional mutagenesis. Mutant GVs were transplanted to the enucleated ooplasm of wild-type oocytes. If the defect was intrinsic to the mutant GV, or defects acquired by chromosomes in the absence of DICER1, then the aberrant phenotype would be expected to be retained in these reconstituted oocytes. Instead, a significant frequency of normalisation was obtained. Out of 50, 30 (60%) reconstituted oocytes underwent PB1E, a frequency similar to non-manipulated and transplant controls, 28 out of 49 (57%) and 34 out of 66 (52%) respectively. Out of 23 examined, 17 (74%) had a normal spindle – two are shown (Fig. 4C and D). This frequency was not as high as in transplant controls, all of which had a normal spindle, 34 out of 34 (100%). In oocytes that did not undergo PB1E, 15 out of 20 (75%) had a normal spindle, again this frequency not as high as in transplant controls, 18 out of 18 (100%). In testing primary oocyte culture conditions, we obtained the highest frequency of PB1E with oocytes of (C57BL/6J × CBA/CaH)F1 (B6CBF1) females, 40 out of 45 (89%). Mutant GVs were therefore transplanted to wild-type enucleated oocytes of this strain to see whether greater frequencies of normalisation would occur. Indeed, 19 out of 24 (79%) reconstituted oocytes underwent PB1E, a frequency similar to that obtained in non-manipulated B6CBF1 controls, 40 out of 45 (89%), and 19 out of 19 (100%) of these possessed a normal spindle. Of oocytes that failed in PB1E, five out of five (100%) had a normal spindle. These results show that, while the mutant GV is responsible at some level for the overall defect, it is likely that mutant ooplasm is the major determinant.

Wild-type GVs were then transplanted to enucleated mutant ooplasm. Only 8 out of 40 (20%) reconstituted oocytes underwent PB1E, a frequency similar to transplant controls, 3 out of 25 (12%). Out of the eight that underwent PB1E, only two (25%) had a normal spindle – two with an abnormal spindle are shown (Fig. 4E and F). This compares with zero out of three (0%) in transplant controls. Of reconstituted oocytes that failed in PB1E, only 7 out of 36 (19%) had a normal spindle, a frequency similar to transplant controls, 2 out of 10 (20%). When wild-type GVs of B6CBF1 oocytes were transplanted to mutant enucleated ooplasm, only 9 out of 30 (30%) reconstituted oocytes underwent PB1E, while 3 out of 9 (33%) of these possessed a normal spindle. Furthermore, out of the 21 oocytes that failed in PB1E, none had a normal spindle. In both of these sets of reciprocal transplantations, there was little, if any, improvement in the frequency of PB1E and normal spindle formation relative to transplant controls and non-manipulated mutant oocytes (DM Mattiske, unpublished observations), thereby confirming that mutant ooplasm is primarily responsible for the meiotic defect. Indeed, some of the cases of normal spindle may have been the result of rescue affected by the carry-over of wild-type ooplasm on transplantation, which was 2–5% of the total oocyte volume. While this amount is relatively small, it is more than the volume of a typical somatic cell and would contain thousands of copies of any highly expressed miRNA (Chen *et al.* 2005).

Discussion

In this study, we have shown that DICER1-conditioned ooplasm is essential for chromosome segregation and completion of the first meiotic division. During meiotic maturation, spindle microtubules capture and orchestrate chromosome movements to form the metaphase plate. The centromere is an essential component of chromosomes where kinetochore assembly occurs, allowing attachment to microtubules, chromosome congression and segregation. We show here that, up to metaphase of the first meiotic division, DICER1-deficient oocytes undergo overtly normal spindle formation and

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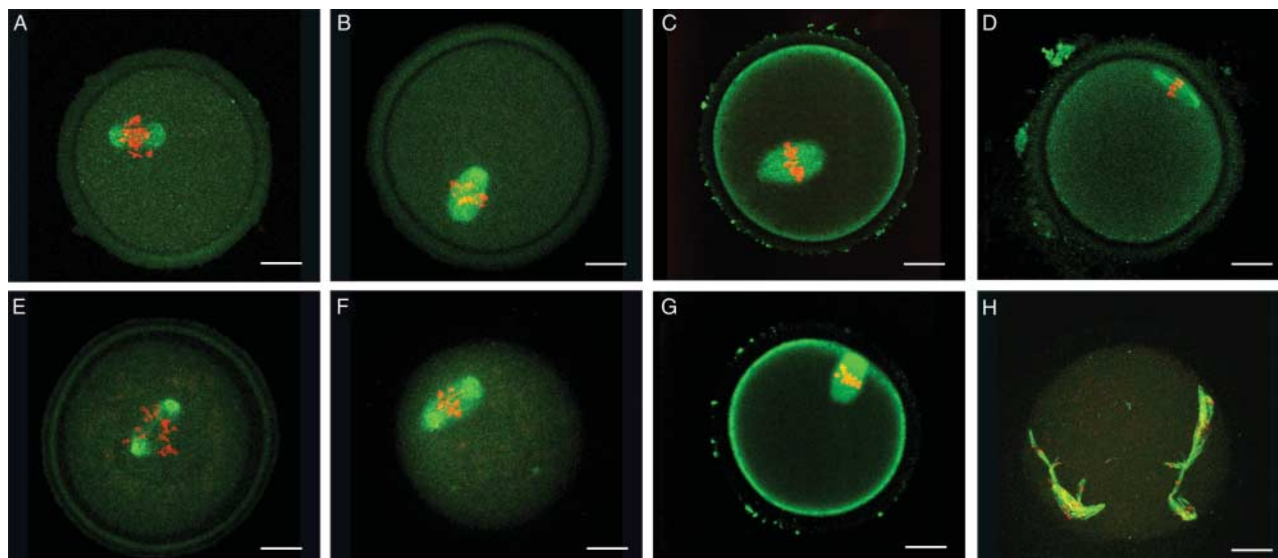

Figure 3 Spindle and chromosome configuration in (A–D) wild-type and (E–H) DICER1-deficient oocytes from GVBD through to metaphase of MII. (A and E) At 2 h after GVBD, both wild-type and DICER1-deficient oocytes had started to form a bipolar spindle and chromosomes had become attached to spindle microtubules. (B and F) Four hours after GVBD, the spindle was complete and chromosomes were congressing to the midline. (C and G) Six hours after GVBD, chromosomes were aligned along the equator of the spindle. The majority (∼70%) of DICER1-deficient oocytes showed a normal spindle and chromosome arrangement; however, some displayed misaligned chromosomes at this stage. (D and H) More than 8 h after GVBD, wild-type oocytes had completed MI, extruded a polar body and chromosomes had realigned on the second metaphase spindle. DICER1-deficient oocytes failed to undergo the metaphase to anaphase transition and formed multiple spindles and misaligned chromosomes.

chromosome congression, indicating that establishment of prometaphase centromere–kinetochore interaction is not a DICER1-dependent mechanism. Furthermore, the development of mutant GVs after transplantation into normal cytoplasm confirms that both kinetochores and centromeres are intact and functioning. Mutant oocytes exhibit normal patterns of CREST staining, although the appearance of unattached centromeres suggests that kinetochore microtubule attachment may be disrupted (Murchison *et al.* 2007). Kinetochores are essential for the initial attachment of chromosomes to the spindle, controlling chromosome movement and maintenance of the spindle assembly checkpoint (SAC). The SAC is a highly conserved mechanism in both mitosis and meiosis that controls the onset of anaphase through the monitoring of spindle integrity and chromosome alignment (Karess 2005). Unattached kinetochores activate the SAC, recruiting a group of checkpoint proteins that inhibit the anaphase-promoting complex (APC/C) and arrests cells at the metaphase stage (Maney *et al.* 1999). The observed defect in DICER1-deficient oocytes after formation of the metaphase plate suggests that the

Table 1 Development of primary oocytes.

Oocyte type	Nimber of oocytes				
	Cultured (number of experiments)	GVBD at 1.5 h	PB1E at 17 h	Normal spindle PB1E	Normal spindle N-PB1E[a]
Control[b]					
Wild type (Wt)	55 (2)	49 (89%)	28/49 (57%)	n.d.[c]	n.d.
Wt-B6CBF1	77 (2)	45 (58%)	40/45 (89%)	n.d.	n.d.
GV→enucleated oocyte					
Control[d]					
Wt→Wt	71 (4)	66 (93%)	34/66 (52%)	34/34 (100%)	18/18 (100%)
Mutant (Mut)→Mut	31 (2)	25 (81%)	3/25 (12%)	0/3 (0%)	2/10 (20%)
Experimental					
Mut→Wt	63 (5)	50 (79%)	30/50 (60%)	17/23 (74%)	15/20 (75%)
Mut→Wt-B6CBFl	39 (3)	24 (62%)	19/24 (79%)	19/19 (100%)	5/5 (100%)
Experimental					
Wt→Mut	51 (5)	40 (70%)	8/40 (20%)	2/8 (25%)	7/36 (19%)
Wt-B6CBFl→Mut	41 (3)	30 (73%)	9/30 (30%)	3/9 (33%)	0/21 (0%)

[a]Oocytes that failed in PB1E. [b]Control for culture conditions; primary oocytes cultured immediately on removal from ovaries. [c]Not done. [d]Control for transplantation procedure.

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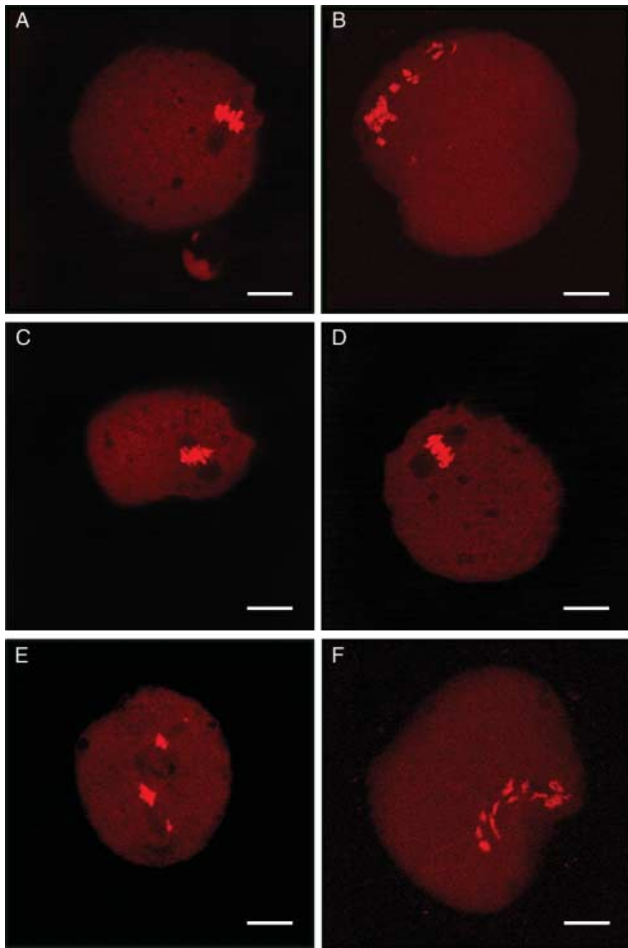

Figure 4 Phenotype of oocytes reconstituted by germinal vesicle transplantation. (A) Control; wild-type GV transplanted into enucleated wild-type ooplasm. (B) Control; mutant GV transplanted into enucleated mutant ooplasm. (C and D) Experimental; mutant GV transplanted into enucleated wild-type ooplasm – mostly normal spindles. (E and F) Experimental; wild-type GV transplanted into enucleated mutant ooplasm – mostly abnormal spindles. Red, DNA stained with PI. Scale bars, 20 μm.

defect lies in either the SAC or in the APC/C. Interestingly, the expression of mRNAs coding several SAC proteins are upregulated in DICER1-deficient oocytes (Murchison *et al.* 2007, Tang *et al.* 2007) and overexpression of several SAC components results in spindle abnormalities and chromosome segregation defects similar to that seen in the mutant oocytes described here (Echeverri *et al.* 1996, Niault *et al.* 2007).

Interestingly, the same abnormal meiotic phenotype induced by the *Zp3-cre* transgene was seen in oocytes when *Dicer1* was excised much earlier in female germ cell development by the *Alpl-cre* transgene. Indeed, many of the excision events are likely to have occurred at the primordial germ cells (PGC) stage. These findings raise the intriguing possibility that DICER1 deficiency does not affect the development of the female germ line at any stage until meiotic maturation. The possibility that PGC proliferation is unaffected by DICER1 deficiency is supported by other studies using this same *cre* transgene for conditional mutagenesis. Near normal numbers of oogonia were observed at 13.5 dpc when *Dicer1* was conditionally mutated (Hayashi *et al.* 2008), whereas the number of mitotic PGCs at 10.5 dpc was severely depleted when the POU domain, class 5, transcription factor 1 (*Pou5f1*) gene was conditionally mutated (Kehler *et al.* 2004). A clearer indication of the effect of DICER1 deficiency in PGCs could be obtained with a *cre* transgene that excises consistently at this stage.

Formation of a normal spindle and completion of the first meiotic metaphase following mutant GV transplants into wild-type enucleated oocytes show that the meiotic defect in DICER1-deficient oocytes is not due to a major deficiency in one or more components of the GV. The prophase nucleus contributes important meiotic components, including those involved in kinetochore specification and assembly. These are the constitutive centromere associated network (CCAN), the foundation of which is centromere protein A (CENPA), and the assemblage of kinetochore protein complexes required for microtubule binding – the KMN network. Other GV components important for kinetochore function are the kinetochore nucleoporins, which, at least in mitotic cells, relocalise to kinetochores after nuclear envelope breakdown (Zuccolo *et al.* 2007, Cheeseman & Desai 2008). With respect to the maintenance of the CCAN, in yeast, the deposition, but not the maintenance, of CENPA is dependent on RNAi-directed heterochromatinisation of regions flanking centromeres (Volpe *et al.* 2003, Fukagawa *et al.* 2004, Motamedi *et al.* 2004, Pal-Bhadra *et al.* 2004, Folco *et al.* 2008). By extrapolation, this could explain why an RNAi-dependent CCAN is relatively unaffected in the present conditional mutagenesis system, as this network could be in a state of stable maintenance well before DICER1 deficiency is induced by the *Zp3-cre* transgene. Another important GV component needed for successful completion of meiotic maturation is cohesin, which has key roles in synapsis, microtubule attachment and chromosome segregation (Petronczki *et al.* 2003, Xu *et al.* 2005). Like CENPA, its deposition is dependent on RNAi-directed centromeric heterochromatinisation (Fukagawa *et al.* 2004). Furthermore, the APC/C, which promotes cohesin degradation and chromosome separation, is present in inhibited form in the meiotic prophase nucleus (Oelschlaegel *et al.* 2005). However, the results here make the important distinction that DICER1 is essential for spindle integrity and meiotic maturation following kinetochore specification and assembly, and is involved in processes within the oocyte cytoplasm.

The ooplasmic defect is likely to involve the loss of miRNAs, which are the end products of DICER1 processing and required for translational regulation of mRNAs in the ooplasm. Indeed, the loss of DICER1

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results in the misregulation of a number of mRNA transcripts in the mature primary oocyte, many of which are candidates for contributing to the observed spindle defects (Murchison *et al*. 2007, Tang *et al*. 2007). However, it is also conceivable that the defect could be related to molecules upstream in the DICER1 pathway, such as double-stranded RNA DICER1 substrate and exportin 5, which transports pre-miRNAs from the nucleus. An abnormally high accumulation of such molecules could be deleterious to ooplasmic processes unrelated to the RNAi system.

In conclusion, our results reveal a previously unknown role for DICER1-dependent factors within the ooplasm of GV oocytes which is essential for the progression of metaphase to anaphase during meiotic maturation. It is likely that several biological processes are being driven by DICER1 to regulate spindle dynamics. Further molecular studies, in conjunction with precise time course experiments through the metaphase to anaphase transition, are required to determine the expression and function of components of the SAC and APC in DICER1-deficient oocytes, and provide insights into the mechanisms regulating mammalian meiotic maturation.

Materials and Methods

Production of Dicer1 conditional mutation

A replacement vector (Mansour *et al*. 1988) was used to make a Cre/*lox*P conditional mutation of *Dicer1* (see Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental/). A 2.06 kb region was flanked by *lox*P sites (floxed) for conditional excision by Cre recombinase. This region contains the last three *Dicer1* exons – nos 25, 26 and 27, encoding 76 out of 213 residues of the second RNAse III catalytic domain and all 57 residues of the double-strand RNA-binding domain (dsRBM) – the translation stop codon and the putative polyadenylation signal. After excision, a null allele (*Dicer1*$^{-}$) is expected because 1) the deleted exons encode highly conserved protein motifs which presumably are indispensable for miRNA and siRNA production, 2) it is unlikely that the truncated RNA will be polyadenylated and exported to the cytoplasm, and 3) if the truncated RNA enters the ribosome, it would be subject to non-stop decay (Frischmeyer *et al*. 2002).

In electroporated embryonic stem cells of *XY* line 2A, mouse strain 129S1/SvImJ (Szabo *et al*. 2006), 5 out of 194 clones assayed were correctly targeted and germ line transmission obtained (see Supplementary Figure 1). For the present studies, the conditional *Dicer1* allele (*Dicer1*^{c}) of one clone (no. 14) was bred to homozygosity and maintained on a mixed genetic background of strains 129S and FVB/NJ. Sequences of all primers used in targeting vector construction and genotype identification are provided in Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/.

Production of oocytes

For conditional mutagenesis, a transgenic line in which the murine zona pellucida glycoprotein 3 (*Zp3*) promoter driving the *cre* coding sequence was used. This excises floxed sequences early in the oocyte growth phase (de Vries *et al*. 2000). In experiments with this transgene, mutant and wild-type females for oocyte production were of genotype *Dicer1*$^{c/-}$, 0/*Zp3-cre* and *Dicer1*$^{c/+}$, 0/0 respectively.

A transgenic line in which the endogenous alkaline phosphatase, liver/bone/kidney (*Alpl*) promoter drives the *cre* coding sequence was also used. Previous studies with this transgene have shown that excision begins in PGCs. In oogonia, at least 60% of cells have undergone excision by 13.5 days post coitum (dpc, day 0.5 dpc = morning of vaginal plug), at the time when meiosis commences, and can progress to higher percentages thereafter (Lomeli *et al*. 2000, Kimura *et al*. 2003, Kehler *et al*. 2004, Tachibana *et al*. 2007, Hayashi *et al*. 2008). Excision probably ceases at 17.5 dpc, the stage when *Alpl* expression is downregulated in germ cells (Cooke *et al*. 1993). In experiments with this transgene, mutant and wild-type females for oocyte production were of genotype *Dicer1*$^{c/c}$, 0/*Alpl-cre* or *Dicer1*$^{c/-}$, 0/*Alpl-cre* and *Dicer1*$^{c/+}$, 0/0 respectively.

Immunohistochemistry and microscopy

Oocytes collected for staining of tubulin were fixed in a microtubule stabilising buffer (0.1 M PIPES, 5 mM $MgCl_2$, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton X-100, 1 μM taxol, 10 U/ml aprotinin and 50% deutronium oxide) for 30 min at 37 °C and washed in PBS with 0.1% BSA (PBSB). Oocytes were blocked in a solution of 2% BSA, 2% serum and 0.01% Triton X-100 overnight at 4 °C. The primary antibody used was β-tubulin (1:5; E7, Developmental Studies Hybridoma Bank, University of Iowa, IA, USA). Samples were incubated in the primary antibody for 1 h at room temperature (RT). After washing in PBSB, samples were incubated with a fluorescent-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:400) for 1 h at RT. Oocytes were counterstained with propidium iodide (PI; 0.25 μg/ml in blocking solution), washed in PBSB and mounted in 1, 4-diaza bicyclo[2.2.2]octane (DABCO).

Oocytes collected for PI staining alone were fixed in 4% PFA for 20 min at RT, stained with PI and mounted in DABCO. Images were collected with a Radiance confocal laser (Bio-Rad).

Quantitative PCR

Approximately 100 oocytes obtained by superovulation were pooled and RNA extracted using the *mir*Vana miRNA Isolation Kit (Ambion). RT reactions for *Dicer1*, and RT and quantitative PCRs for actin, beta, cytoplasmic (*Actb*) transcripts were carried out as previously described (Lefevre & Mann 2008). Primers used; *Dicer1*, internal to the floxed DNA, 5′–3′, CAT TTT TGA GTC TGT TGC TGG TGC and GGC AGT TTC TGG TTC CAT CTC G, *Actb* (Carmell *et al*. 2007). To confirm that a loss of DICER1 activity results in a reduction in the DICER1-dependent small RNA population as previously shown

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(Murchison *et al.* 2007, Tang *et al.* 2007), the expression of two small RNAs was analysed. miRNAs *Mirn16-1* and *Mirn30b*, two miRNAs known to be expressed at high levels in oocytes (Murchison *et al.* 2007), were assayed using the TaqMan MicroRNA RT Kit and TaqMan MicroRNA assays (Applied Biosystems). Assays were performed in triplicate. miRNA expression was normalised to *Actb* and expressed relative to the amount of *Mirn16-1* in wild-type oocytes.

GV transplantation

GV transplantation was carried out essentially as described (McGrath & Solter 1983, Liu *et al.* 1999, Takeuchi *et al.* 1999; see Supplementary Figure 2, which can be viewed online at www.reproduction-online.org/supplemental/) using inactivated Sendai virus (Cosmo Bio Co., Tokyo, Japan) for karyoplast fusion. Mice of 8–16 weeks of age were used as oocyte donors. Following micromanipulation, oocytes were placed in culture, then after 100 min were scored for karyoplast fusion – which occurred in 100% of manipulated oocytes – and GVBD. Those undergoing GVBD were cultured for a further 16 h, scored for extrusion of a polar body and processed for confocal microscopy.

Oocyte culture

GV oocytes were collected from ovaries of 5- to 6-week-old mice following superovulation as per GV transplantation procedure. Denuded oocytes were placed into culture with $KSOM_g^{AA}$ (Biggers *et al.* 2005), except that L-glutamine was replaced by alanyl-glutamine, and observed until GVBD. Oocytes were removed from culture, fixed at intervals of 2, 4, 6 and >8 h following GVBD and processed for immunohistochemistry.

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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