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Letter to the Editor

Recurrent pregnancy loss in mice lacking the X-linked *Ccnb3* gene†

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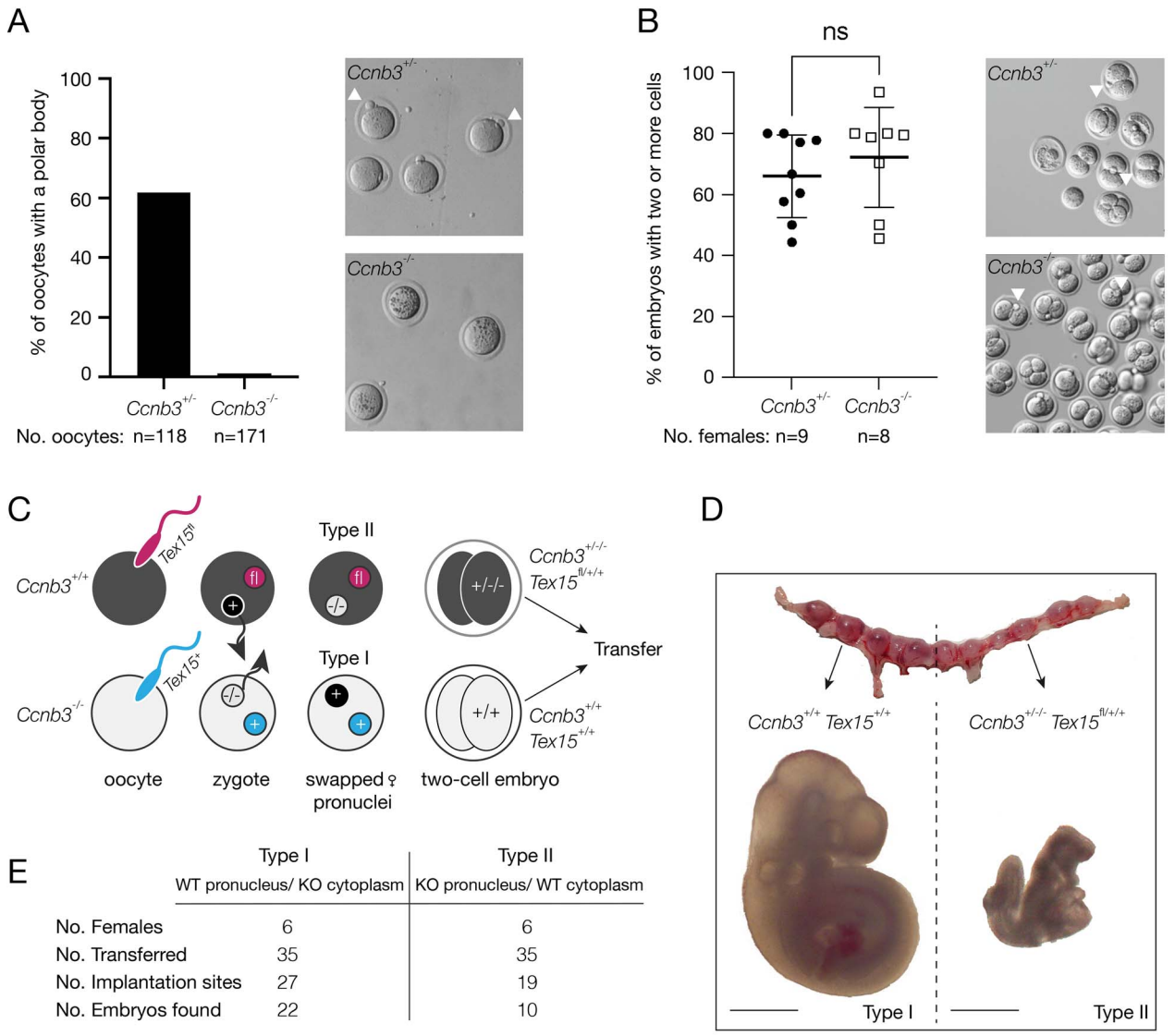
Dear Editor,

Cyclins and cyclin-dependent kinases regulate both mitotic and meiotic cell cycles [1]. Most cyclins are ubiquitously expressed; however, a few are unique to the germline. Cyclin B3 (CCNB3)—an evolutionarily conserved type B cyclin—is meiosis-specific in mammals. In *Drosophila* and *Caenorhabditis elegans*, CCNB3 is required for early embryonic divisions. In mice, *Ccnb3*—an X-linked gene—is required for female fertility but dispensable for male fertility. Specifically, RNAi and knockout studies have shown that mouse CCNB3 is required for metaphase I to anaphase I transition in oocytes [2–4]. *Ccnb3*-deficient oocytes fail to extrude the first polar body and are arrested at metaphase I, due to a failure in degradation of securin (inhibitor of the protease separase) and CCNB1 (a component of the metaphase promoting factor). Upon fertilization, *Ccnb3*-deficient oocyte skips the first meiotic cell division (Meiosis I) but extrudes one polar body through segregation of sister chromatids (Meiosis II), resulting in non-viable triploid embryos [3]. By generation and study of an independent *Ccnb3* knockout mouse line, we have confirmed these previous findings and further explored the embryonic requirement for *Ccnb3*.

We disrupted *Ccnb3* in mice using the CRISPR/Cas9 approach. Two guide RNAs targeted the intronic regions flanking exons 6 and 7 of *Ccnb3* (Supplementary Figure S1A). $Ccnb3^{-/Y}$ males exhibited normal histology of testis, testis weight, sperm count, and were fertile (Supplementary Figure S1B–D). $Ccnb3^{-/-}$ females displayed normal ovarian histology but were infertile (Supplementary Figure S1E). Although 61.9% oocytes from $Ccnb3^{+/-}$ females extruded a polar body, only 1.2% oocytes from $Ccnb3^{-/-}$ females did so (Figure 1A). We next tested the competency of *Ccnb3*-deficient oocytes for fertilization by quantifying progression to the two-cell stage of embryogenesis 36 h after mating with wild-type males. *Ccnb3*-deficient oocytes were fertilized and extruded a single polar body. The resulting zygotes progressed to the two-cell stage in the same percentage as wild-type zygotes, showing that CCNB3 is dispensable for fertilization (Figure 1B). Although $Ccnb3^{-/-}$ females readily became pregnant, their pregnancies were never carried to term. To further examine the development of the embryos derived from *Ccnb3*-deficient oocytes, we mated $Ccnb3^{+/-}$ and $Ccnb3^{-/-}$ females to wild-type males and collected embryos at 9.5 days after fertilization (E9.5). Examination with light microscopy revealed malformations of embryos conceived to $Ccnb3^{-/-}$ females, indicating a disruption in embryogenesis consistent with other mouse models of triploidy [5]. This result suggests that the embryos derived from $Ccnb3^{-/-}$ females were able to develop to the blastocyst stage and implant.

Mature oocytes are transcriptionally silent. The presence of the cytoplasmic polyadenylation element (UUUUAU; [6]) upstream of the polyadenylation signal sequence (AAUAAA) in the *Ccnb3* transcripts from mouse, human, and other species suggest that *Ccnb3* might be translationally dormant in immature oocytes (Supplementary Figure S1F). The initial stage of embryogenesis is driven by stored maternal transcripts and proteins in the oocyte, some of which are required for zygotic genome activation (ZGA). ZGA is a hallmark of the maternal to zygotic transition, during which maternal RNAs driving cellular processes in the oocyte and zygote are degraded, whereas the zygotic genome becomes transcriptionally active to take over the production of essential proteins. Maternal transcripts involved in ZGA are characterized by high abundance in the oocyte and pre-ZGA embryo but a sharp decrease in abundance after ZGA. Mouse and human *Ccnb3* genes exhibit such a characteristic expression pattern [7], and furthermore, depletion of *Ccnb3* in *Ciona intestinalis* causes precocious ZGA [7].

We reasoned that if stored *Ccnb3* transcripts were important for ZGA in mice, their depletion would disrupt embryogenesis. To distinguish between cytoplasmic and nuclear contributions of *Ccnb3*, we performed pronucleus swapping experiments (Figure 1C; [8]). In these experiments, wild-type females were mated with $Ccnb3^{+/Y}$ $Tex15^{fl/fl}$ males, and $Ccnb3^{-/-}$ females were mated to $Ccnb3^{+/Y}$ $Tex15^{+/+}$ males. The $Tex15^{fl}$ and $Tex15^{+}$ alleles (autosomal) were used to discern between paternal pronuclei. Upon formation of pronuclei in one-cell embryos, the maternal pronuclei were swapped between the embryos derived from *Ccnb3*-deficient oocytes and those from wild-type oocytes. The swap resulted in two types of embryos: (I) embryos with a wild-type *Ccnb3* pronucleus and *Ccnb3*-deficient cytoplasm; and (II) embryos with a maternal $Ccnb3^{-/-}$ pronucleus and wild-type *Ccnb3* cytoplasm (Figure 1C). Types I and II embryos were transferred to the right and left sides, respectively, of the uterine horns of pseudopregnant females and allowed to develop to E9.5 (Figure 1D). An equal number (35 each) of both types of embryos were transferred to six females. Embryos were then harvested for microscopy and genotyping (Supplementary Figure S1G and Supplementary Materials). Since *Ccnb3*-deficient oocytes only extruded one polar body, the *Ccnb3*-deficient pronucleus was diploid ($Ccnb3^{-/-}$) and the resulting embryos were triploid. Therefore, we anticipated that embryos with $Ccnb3^{-/-}$ pronuclei would degenerate regardless of the presence of *Ccnb3* transcripts in the cytoplasm, because of triploidy. A total of 22 type I embryos were recovered; these embryos were morphologically normal. In contrast, only 10 type II embryos were recovered



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	Type I WT pronucleus/ KO cytoplasm	Type II KO pronucleus/ WT cytoplasm
No. Females	6	6
No. Transferred	35	35
No. Implantation sites	27	19
No. Embryos found	22	10

Figure 1. Recurrent pregnancy loss in mice lacking CCNB3. (*A*) Percentage of ovulated oocytes from 8-week-old *Ccnb3*$^{+/-}$ and *Ccnb3*$^{-/-}$ females with a polar body. Arrowheads indicate polar bodies. The oocytes analyzed were pooled from 6 *Ccnb3*$^{+/-}$ females and 7 *Ccnb3*$^{-/-}$ females. (*B*) Percentage of embryos at the two-cell stage 36 h after mating. Representative mages of embryos are shown. Arrowheads indicate polar bodies. The total number of embryos analyzed: 227 from 9 *Ccnb3*$^{+/-}$ females and 181 from 8 *Ccnb3*$^{-/-}$ females. NS, statistically non-significant, by Student's *t*-test. (*C*) Schematic of maternal pronuclei (black and white) swap experiment. Paternal pronuclei (colored) are either *Tex15*^{fl} or *Tex15*$^{+}$ (wild type). Note that paternal pronuclei were not swapped. Detailed procedure is described in Supplementary Material. (*D*) Images of uterine horns collected from a recipient female at E9.5. On the left are implantation sites of type I embryos. On the right are implantation sites of type II embryos. The resultant E9.5 embryos are shown below their respective sides of the uterine horn. Genotypes are shown. Scale bar, 1 mm. (*E*) Development of pronucleus swap embryos.

after 9.5 days and all of them were abnormal in size and shape (Figure 1E). These results demonstrate that the triploidy of embryos derived from *Ccnb3*-deficient oocytes is the primary cause of embryo death, i.e., such embryos can be rescued with euploid nuclei, whereas cytoplasmic *Ccnb3* transcript is dispensable for ZGA and embryo development.

Our findings underscore the requirement for CCNB3 in female meiosis and suggest that ZGA-specific functions of CCNB3 observed in invertebrate species are not likely conserved in mammals. The *Ccnb3*-deficient mouse model is similar to a human infertility condition—recurrent pregnancy loss (RPL). RPL is characterized by two or more consecutive pregnancy losses in the first trimester. Recent case studies have identified *CCNB3* mutations as genetic causes of RPL in women [9, 10]. Because *CCNB3* is X-linked and CCNB3 is dispensable for male fertility, deleterious mutations may be propagated through males and heterozygous females. Clinically, mutations in *CCNB3* are likely to be frequent underlying genetic causes of RPL in humans.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Conflict of interest

The authors declare they have no conflicts of interest.

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