ARTICLE
Journal of Cellular Biochemistry 117:1806–1812 (2016)

Journal of Cellular Biochemistry

Presence of the Paternal Pronucleus Assists Embryo in Overcoming Cycloheximide Induced Abnormalities in Zygotic Mitosis

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ABSTRACT

After fertilization, the maternal and paternal chromosomes independently proceed through pronuclear formation. These chromatin reconfigurations occur within a shared cytoplasm thus exposing both gametes to the same factors. Here, we report that continuous cycloheximide [40 μg/mL] treatment of parthenogenotes, androgenotes, and ICSI embryos reveals ORC2 pronuclear instability in the maternal (MPN) but not the paternal pronucleus (PPN). When released from CHX after 8 h, the MPN can recover ORC2 and proceed through replication, however, parthenogenotes encounter severe mitotic defects while both ICSI embryos and androgenotes are able to recover and develop at significantly higher rates. Taken together, these data suggest cycloheximide treatment promotes an environment that asymmetrically affects the stability of ORC2 on the MPN, and the ability of the MPN to develop. Furthermore, the presence of the PPN in the zygote can ameliorate both effects. These data suggest further evidence for crosstalk between the two pronuclei during the first cell cycle of the embryo. J. Cell. Biochem. 117: 1806–1812, 2016.

KEY WORDS: EMBRYO; DEVELOPMENT; REPLICATION; CYCLOHEXIMIDE; ORC2

A growing body of research continues to support the fact that the maternal and paternal nuclei behave asymmetrically in the zygote thereby each uniquely influencing embryonic development [Barton et al., 2007; Gawecka et al., 2013; Jiang et al., 2013; Liu et al., 2014]. While it is known that the successful transmission of spermatozoon genetic information to the oocyte is critical for proper development [D'Occhio et al., 2007; Gawecka et al., 2013], it is important to observe that many chromatin-associated features are acquired de novo by the paternal genome after fertilization. This implicates the sperm-to-pronuclear transformation in the zygote as a unique opportunity to investigate how relatively naïve chromatin interacts with zygotic cytoplasm [Ward et al., 2000; Loppin et al., 2005; Probst et al., 2010]. One such de novo acquisition by the paternal genome is the establishment of pre-replication complex (pre-RC) proteins to potential origins [Gillespie and Blow, 2000; Gillespie et al., 2001].

Pre-RC formation is initiated by the ORC(2-5) proteins and concludes with the loading of the MCM(2–7) helicase complex during the M-G_1 transition, thereby distinguishing the site as competent for replication [Ghosh et al., 2011; Yeeles et al., 2015]. This process is referred to as licensing. During gametogenesis, sexual dimorphic expression of replication factors in the testes, and ovaries have been observed [Eward et al., 2004], however, this might be expected since female gametes enter meiosis while paternal gametes arrest thereby presenting them at different stages of the cell-cycle upon fertilization. In consideration of these observations, it is reasonable to question how licensing progresses in the zygotic environment.

We have previously shown that ORC2 interacts very differently in the sperm and oocyte chromatin during the first stages of embryonic development when the two pronuclei are formed, but appeared to be similar after pronuclear formation [Ortega et al., 2012]. In this report, we tested whether we could indentify any asymmetry in ORC2 behavior between the two pronuclei by inducing stress with cycloheximide (CHX) treatment. Here, we report that the PPN and MPN had different ORC2 profiles upon

Conflicts of interest: The authors declare no conflict of interest.
Grant sponsor: NIH; Grant number: HD060722.
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Manuscript Received: 17 December 2015; Manuscript Accepted: 4 January 2016
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 5 January 2016
DOI 10.1002/jcb.25480 • © 2016 Wiley Periodicals, Inc.

CHX recovery. We also found that upon release from CHX treatment, embryos without the paternal genome encountered severe zygotic mitosis defects, and arrest at the one-cell stage.

MATERIALS AND METHODS

ANIMALS

B6D2F1 (C57BL/6N X DBA/2) mice were obtained from National Cancer Institute (NCI) (Raleigh, NC) and Charles River Laboratories (CRL). Mice were kept in accordance with guidelines by the Laboratory Animal Services at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEF publication no. [NIH] 80–23, revised 1985). The protocol for animal handling and the treatment procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

COLLECTION OF GAMETES

The luminal content of the caudal epididymides ~8-week-old mice was extracted and suspended in HCZB for 30 min at 37°C under 5% CO_2. Swim-up sperm were selected for intracytoplasmic sperm injection (ICSI). To obtain metaphase-II oocytes, mature females, 8–12 weeks old, were induced to superovulate with i.p. injections of 5 IU eCG and 5 IU hCG given 48 h apart. Oviducts were removed 14–15 h after hCG injection and placed in Ca^{+2}-free CZB containing 0.1% of bovine testicular hyaluronidase (300 USP units/mg) to disperse cumulus cells. Cumulus-free oocytes were washed with Ca^{+2}-free CZB medium and processed accordingly for the desired cell type.

INTRACYTOPLASMIC SPERM INJECTION

ICSI was carried out as described by Szczygiel and Yanagimachi [Szczygiel and Yanagimachi, 2003]. Briefly, spermatozoa were prepared for ICSI by mixing with 12% w/v polyvinyl pyrollidone (PVP, 360 kDa). Single sperm heads were separated from the midpiece by applying one or more piezo pulses. After discarding the midpiece and tail, the head was redrawn into the pipette and injected immediately into an oocyte. ICSI was performed using Eppendorf Micromanipulators (Micromanipulator TransferMan, Eppendorf, Germany) with a Piezo-electric actuator (PMM Controller, model PMAS-CT150, Prime Tech, Tsukuba, Japan). After ICSI, oocytes were cultured in CZB [Chatot et al., 1989] at 37°C in 5% CO_2 or with CZB supplemented with various treatments.

ENUCLEATION OF METAPHASE II OOCYTES AND DONOR SPERM INJECTION FOR ANDROGENOTES

M-II oocytes were transferred into a droplet of M2 Media (Millipore, Billerica, MA) containing 5 g/mL cytochalasin B, which had previously been placed in the operation chamber on the microscope stage. Oocytes undergoing microsurgery were held with a holding pipette and the zona pellucida following the application of several piezo-pulses to an enucleation pipette. The M-II chromosome–spindle complex (identifiable as a translucent region) was aspirated into the pipette with a minimal volume of oocyte cytoplasm. After enucleation of all oocytes in one group (10–15 min), were transferred into cytochalasin B-free KSOM (Millipore) and cultured for up to 2 h at 37°C under 5% CO_2. ICSI was then performed in enucleated oocytes with fresh B6D2F1 male swim-up spermatozoa at room temperature. Injections were performed as previously described.

ANTIBODIES

Polyclonal goat anti-ORC2 (Santa Cruz Biotechnology Santa Cruz, CA, C-18, cat# sc-13238) and monoclonal mouse-MCM7 (Santa Cruz Biotechnology, G-7, cat# 46687) primary antibodies were used. Secondary antibodies included Alexa Fluor 488 donkey anti-goat, Alexa Fluor 546 donkey anti-mouse, and Alexa Fluor 546 rabbit anti-goat (Invitrogen, Carlsbad, CA). The Click-iT EdU Alexa Fluor 488 and 555 HCS Assay kits were used depending on the desired combination, according to the manufacturer's protocol (Invitrogen, cat# C10350 and #C10352 respectively) to verify DNA synthesis.

TREATMENTS

Cycloheximide. All CHX treatments were performed using CZB supplemented with 40 μg/mL of CHX. To release cells from CHX treatment, a rinse, and two 5 min CZB washes were performed followed by transfer to fresh CZB.

Morpholinos. An antisense morpholino was purchased from GeneTools (Philomath, OR) targeting the major *Orc2*-mRNA transcripts (a and b) with the sequence 5′-TTTCCTTTAACTGCAGAGTGCT-3′ which included a 3′ fluorescein. The control morpholino 5′-CCTCTTACCTCAGTTACAATTTATA-3′ also had a 3′ fluorescein. The negative control morpholino targets a human beta-globin intron mutation that should have little impact on phenotype. To determine the optimal amount of morpholino for microinjection into the mouse embryo, embryos were injected with increasing concentrations (0.2 mM, 5.0 mM, and 10 mM) of the control morpholino, and their development was followed. Since injection of 0.2 mM control morpholino resulted in no obvious complications to the embryo, this concentration was used for testing the *Orc2*-mRNA targeting morpholino. For preparation, morpholinos were resuspended in PCR grade water and heated for 15 min at 65°C prior to injection. M-II embryos were injected with either *Orc2*-mRNA targeting or control morpholino and permitted to rest for 1 h prior to ICSI.

SALT RESISTANCE

To test DNA binding affinity, embryos were washed in ice-cold Ca^{+2}/Mg^{+2} free PBS containing 0.5 mM PMSF, 10 μg/mL of leupeptin, and aprotinin (modified ice-cold cytoskeleton buffer, mCSK) for 3 min on ice followed by washing in mCSK with 0.5% Triton X-100 and 100 mM NaCl for 2 min on ice then performing a final wash in mCSK for 3 min on ice. Cells were then fixed according to the ICC protocol described below.

IMMUNOCYTOCHEMISTRY

Cells were cultured in CZB and fixed in 4% paraformaldehyde for 30 min at room temperature. Paraformaldehyde stock solutions were prepared by diluting a 16% stock solution (Alfa Aesar, Ward Hill, MA, stock# 43368) before each experiment. After fixing, cells were rinsed once and washed twice in 0.1% Tween/PBS for 10 min (PBSw). Immunocytochemistry (ICC) was performed as previously described

[Ortega et al., 2012]. Primary antibody was used at a 1:300 dilution and secondary antibody at 1:1000 dilution. Cells were mounted with ProLong Gold antifade reagent containing DAPI (Invitrogen, cat# P-36931) and analyzed with an FV1000-IX81 confocal microscope from Olympus (Shinjuku, Japan) using Fluoview v. 2.1 software within a week of preparation.

ANALYSIS OF ORC2 AND MCM7 LOCALIZATION

Each stage of development and treatment for each cell type analyzed were performed in at least three separate experiments with the total n = values described in the Figure descriptions. After confocal analysis, images were processed with Imaris 8.0 (Bitplane, Belfast, U.K.). Briefly, the total sum ORC2 and MCM7 localization as obtained by 0.25 μm confocal series and were processed using the Surfaces command. The surface area detail was set to 0.500 μm with a background subtraction of 0.800 μm. From this analysis, PN localization was then used to calculate relative qualitative measurements. For DAPI signals, the surface area detail was set at 0.125 μm. The total sum intensities were assessed for significance with GraphPad Prism 6.0 using one-way ANOVA with Tukey post-hoc test. For significance: no significance = $P > 0.05$, $^{*} = P \leq 0.05$, $^{**} = P \leq 0.01$, $^{***} = P \leq 0.001$, $^{****} = P \leq 0.00001$.

RESULTS

ANDROGENOTES AND ICSI EMBRYOS, BUT NOT PARTHENOGENOTES CAN RECOVER FROM CHX TREATMENT TO PROGRESS TO THE 2-CELL STAGE

Before testing the symmetry of the responses by the MPN and PPN to CHX stress, we first needed to determine if the early development of embryos was affected differently by CHX depending on the genome complement they contained. Time point zero (T_0) for each embryo type began when the oocyte was activated with $SrCl_2$. Parthenogenotes, androgenotes, and ICSI embryos all demonstrated similar temporal development as marked by entry into zygotic mitosis by 18 h post activation. When translation is inhibited by CHX for up to 8 h and then released, most of the ICSI embryos and androgenotes continued to develop to the 2-Cell stage (Fig. 1). However, we were surprised to find that very few parthenogenotes (4/58) made it past metaphase. Because of this discrepancy, we tested whether different incubation times with CHX would allow parthenogenotes to develop. Parthenogenotes activated without CHX treatment and permitted to develop for as little as 2 h prior to introducing CHX provided these cells with enough time to generate material to support some DNA synthesis (Supplemental Fig. S1). Parthenogenotes that are only treated for 4 h in CHX before release are able to rapidly recover the ability to replicate DNA and develop beyond two-cell (16/18) unlike those released after 8 h. These data suggest that the presence of the PPN in the embryo is required for a robust recovery from CHX treatment.

ORC2 ASYMMETRICALLY DEPLETES FROM THE MPN DURING CHX TREATMENT

Having indentified asymmetry in the developmental response of embryos with and without the paternal genome, we next tested whether there was a similar asymmetry in the response of ORC2 binding to DNA in the pronuclei with CHX treatment. We noticed two things with respect to ORC2 accumulation in the pronuclei; PPN tended to have more ORC2 than MPN, and CHX treatment generally decreased ORC2 accumulation in all types of pronuclei (Fig. 2A). Parthenogenotes incorporated more ORC2 than any other genome complement tested (Fig. 2B). Parthenogenote MPNs had the highest levels of ORC2, which decreased with CHX treatment, which in turn have more ORC2 than CHX treated ICSI embryo MPN.

Androgenotes were difficult to assess at this time point as the sperm was likely to be pulled to the periphery of the embryo and in some cases were extruded (data not shown). However, under CHX treatment of androgenotes the PPN was more likely to be in the center of the egg and therefore the PPN signal could be more accurately quantified (Fig. 2B). There was no difference between the relative amounts of ORC2 detected in CHX treated parthenogenotes and androgenotes.

We next tested one function of ORC2 in CHX treated embryos, binding to DNA. Extracting the embryos with 100 mM NaCl prior to fixation removes unbound ORC2 [Donovan et al., 1997; Ortega et al., 2012]. We found that there was a much larger difference between the levels of DNA bound ORC2 in the PPN versus the MPN. Salt washed androgenotes contained significantly more ORC2 than salt extracted parthenogenotes, and PPN contained much higher levels of DNA bound ORC2 than MPN in ICSI generated embryos (Fig. 2C). These data suggest that the PPN has a much greater ability to retain DNA bound ORC2 than the MPN, and that, unlike early embryonic development, the presence of the male genome does not confer additional resilience to CHX treatment to the female genome.

Interestingly, this effect was not seen for DNA bound MCM7, the final protein that binds during pre-replication complex assembly during licensing. MCM7 levels appeared similar by ICC and did not change with CHX treatment (Fig. 3). There were some differences between MPN and PPN, but these were not as large as with ORC2 (Fig. 3B). Moreover, the salt resistant (DNA bound) MCM7 was very similar between MPN and PPN in all three types of embryos (Fig. 3C). This suggests the male and female pronuclei are equally licensed despite the fluctuations observed in ORC2. Thus, while the CHX treated androgenotes appear to import MCM7 more abundantly than the CHX treated parthenogenotes, there is no detectable difference in the ability to retain and bind this cargo to the DNA.

ORC2 IS ASSOCIATED WITH SEVERE METAPHASE DEFECTS DURING ZYGOTIC MITOSIS OF CHX RELEASED PARTHENOGENOTES

Because of the unique arrest of CHX treated parthenogenotes at zygotic metaphase, we questioned whether ORC2 might play a role in this. We observed that under normal conditions ORC2 asymmetrically aligns on each side of the metaphase chromatin (Fig. 4), as observed in ICSI embryos and HeLa cells [Prasanth et al., 2004; Ortega et al., 2012]. However, in parthenogenotes with metaphase defects, ORC2 positioning was disrupted and diffuse, and coincided with the disorganized chromosome metaphase plate (Fig. 4B). These data suggest a potential non-replicative role for ORC2 in metaphase segregation that is disrupted during the first 8 h of CHX treated parthenogenotes. Supporting this suggestion, we found that ICSI generated embryos treated with Orc2 morpholinos also arrested at zygotic metaphase (Supplemental Figs. S2 and S3).

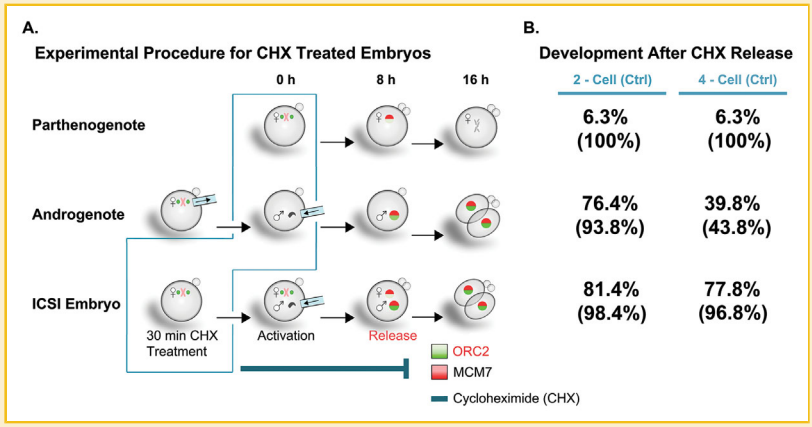

Fig. 1. Development of embryos released from CHX after 8 h is facilitated by the presence of sperm. (A) The experimental design for determining the affects of CHX treatment on different embryo types is depicted in this schematic. Control parthenogenotes were activated with 10 mM $SrCl_2$ which marked 0 h (T_0) on our time progression for embryo development. T_0 for all other embryo types was also initiated with the exposure to $SrCl_2$, except ICSI embryos for which T_0 was counted as the time of injection. All cell types were analyzed under various conditions at 8 h post activation because this is the time when the average population of these cells begin DNA synthesis, as measured by the initiation of EdU incorporation. The incorporation of EdU served as a known developmental marker to measure the effects of our treatments. After 8 h, cells could either be continuously inhibited or released and observed for their ability to recover. Each of the cell types were tested for their ability to recover from 8 h of CHX treatment and their development was monitored until at least 4-cell stage. The 8 h time point was chosen for release because these cells are typically in early S-phase. All cell types were able to significantly reach 2-cell stage except for parthenogenotes, which almost without exception arrested at one-cell stage. Of the remaining cell types, only androgenotes significantly failed to reach 4-cell stage. (B) Percentatges of embryos that reached 2-Cell and 4-Cell stages. Significance was measured by one-way ANOVA with Tukey post-hoc test, *P* value of <0.05. CHXreleased (ctrl) n = ICSI-58(42), parthenogenotes-58(13), androgenotes-49(28).

DISCUSSION

One of the most intriguing features in the mouse zygotic embryo model is the asynchronous development that occurs as the maternal and paternal genomes interact with a shared cytoplasm. Our previous studies suggested that the maternal DNA might be partially licensed prior to fertilization while the sperm received these factors from the embryo [Ortega et al., 2012; Nguyen et al., 2015]. The data presented here further suggests that while the MPN and PPN are exposed to the same oocyte cytoplasmic factors, the manner in which they interact with them are different. While we began this work to investigate potentially different DNA licensing responses to stress by the two pronuclei, we found that after release from CHX-induced stress, embryos that contain PPN are significantly more likely to pass through zygotic metaphase than those without. The data suggest, as discussed below, that a non-replicative role in chromosome segregation is one potential interpretation for explaining how differential responses in ORC2 DNA binding might be related to the differences in early embryonic development.

ORC2 IS MORE STABLE IN PPN THAN MPN IN RESPONSE TO CHX

MPN were much more susceptible to the loss of ORC2 in response to CHX than PPN and this depletion was greatest in CHX treated ICSI embryos (Fig. 2B). This difference in asymmetric depletion was further emphasized when testing for DNA bound ORC2 where we found that MPN had little (parthenogenotes) to almost no ORC2 (ICSI MPN) bound to the DNA, while PPN of both androgenotes and ICSI-generated embryos retained ORC2 on the DNA (Fig. 2C). While topology has been suggested to affect ORC recruitment [Remus et al., 2004], our observations would further suggest that once recruited PPN utilize an alternative mechanism for retaining ORC2. Asymmetric retention of ORC2 could reflect the state of the chromosomes since pre-RC assembly has been shown to affect nucleosome remodeling [Belsky et al., 2015], which are being established during paternal pronuclear formation.

We previously suggested that most, if not all, the DNA in the zygote was licensed by S-phase and that the continued presence of DNA bound ORC2 on late stage pronuclei probably represented another function [Li and DePamphilis, 2002; Ortega et al., 2012]. Our current data support this, since while ORC2 displayed dynamic behavior on the MPN when compared to the PPN under CHX treatment, the overall ability to license DNA in the zygote, as detected by salt resistant MCM7, was relatively the same for most chromatin types tested (Fig. 3C). Furthermore, all three types of embryos tested were able to replicate their DNA when released. These data suggest that the differences in ORC2 behavior between the PPN and MPN may be related to another function of ORC2 or an ORC2 associated protein.

Although, ORC2 localized to each type of PN tested while under CHX treatment after activation, these data agree with previous reports indicating ORC2 is present in M-II oocytes [Murai et al., 2010], however, the asymmetric pronuclear depletion observed further suggest an additional level of maintenance that distinguishes the way this protein interacts with the MPN and the PPN. Taken together, these observations suggest the recruitment and maintenance of ORC2 is not only dependent on the state of the cytoplasm but also dependent on the state of the chromatin.

EMBRYOS CONTAINING PPN RECOVER FROM CHX TREATMENT BETTER THAN PARTHENOGENOTES

When parthenogenotes were released from CHX treatment after 8 h they overwhelmingly encountered metaphase defects and stalled at the one-cell stage, while androgenotes and ICSI generated embryos

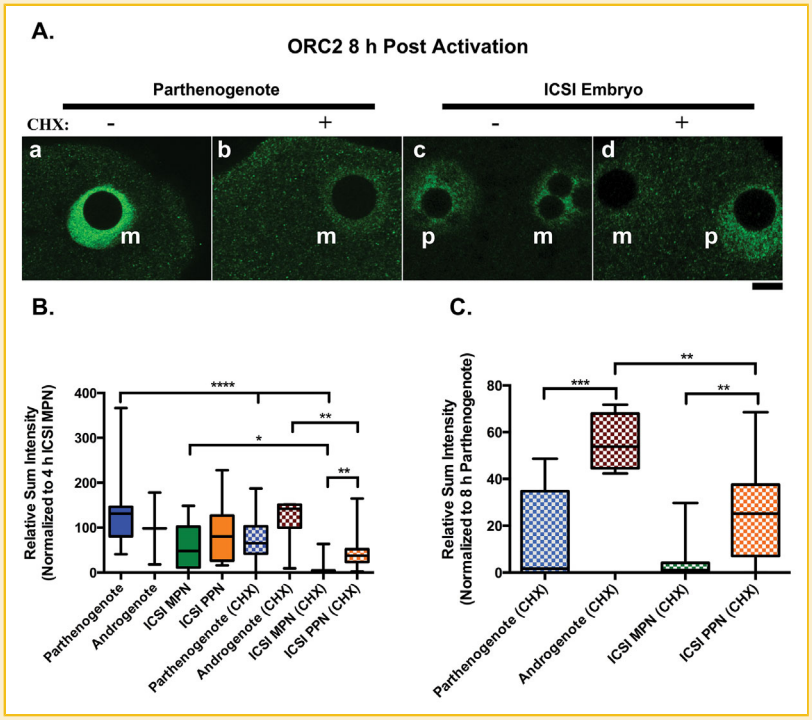

Fig. 2. Effect of CHX on pronuclear ORC2 loading during early zygotic development. (A) Examples of ORC2 levels in the pronucleis of parthenogenotes (a and b) and ICSI generated embryos (c and d) without (a and c) and with (b and d) CHX treatment. All images shown here are single plane 0.25 μm confocal slices. Scale Bar = 5 μm (m = maternal pronucleus, p = paternal pronucleus). (B) Eight hours is typically the time point that parthenogenotes and ICSI embryos begin DNA synthesis. These groups were normalized to 4 h ICSI MPN to emphasize the loading process that takes place during G_1. The duration of treatments made it technically difficult to obtain androgenotes because the embryo sometimes extrudes foreign DNA. From left to right for the groups shown n = 20, #2, 11, 11, 34, 7, 52,52. (C) Samples from each group were washed in 100 mM NaCl prior to 4% PFA fixation in order to determine DNA binding affinity. These cells were normalized to 8 h parthenogenotes in order to highlight the affects of CHX on binding affinity for each chromatin type at a given time point. From left to right for the groups shown n = 17, 4, 17, 17. For Figure 2B and C, significance was measured by one-way ANOVA with Tukey post-hoc test, *P* value of <0.05. Significance for all comparisons not shown were high (****) except for parthenogenote versus ICSI PPN in (B) which was (*) and parthenogenote versus androgenote (CHX) in (C) which was (*). The number of untreated androgenotes at this time point were difficult to quantify because they tended to be pulled toward the periphery of the embryo or extruded and therefore the ICC signal could not be cleanly isolated. n represents the number that could be counted but significance could clearly not be drawn from this.

progressed to the 2-Cell stage. This effect was not observed in previous studies using lower doses of cycloheximide [Moos et al., 1996]. Because ICSI generated embryos did contain one MPN, these data suggest that the presence of the PPN renders the stability of the ICSI embryo, rather than the presence of the MPN causing the instability in the parthenogenote. While the mechanism for this remains unknown, the data suggest that some crosstalk between the two pronuclei occurs so that in ICSI generated embryos the PPN provides some stability for the embryo to at least pass through zygotic metaphase. Evidence for this type of crosstalk between the two pronuclei of mammalian embryos has been previously reported [Barton et al., 2007; Grenier et al., 2010; Gawecka et al., 2013].

ZYGOTIC METAPHASE STALL IN PARTHENOGENOTES RELEASED FROM CHX

The metaphase abnormalities encountered by CHX released parthenogenotes suggest that some component other than ORC2 which is normally required for parthenogenetic division is synthesized during the first 8 h, and if this does not occur parthenogenotes will not be able to appropriately self organize along the metaphase plate. The observation that ORC2 follows abnormal spindle pole positioning (Fig. 4) suggests that under normal conditions, this component may associate with ORC2 for zygotic division. In the absence of translation during the first 8 h, it appears the normal development of the PPN is able to prevent the zygote from one-cell block, which suggests the sperm contributes a translation-independent factor that assists in division. In humans, sperm donate the centrosome, and ORC2 has previously been linked to centrosomes in somatic cells [Prasanth et al., 2004; Hemerly et al., 2009], however, in mice, sperm have long been considered to lack this paternally donated centrosome [Manandhar et al., 2005]. Interestingly there may be one candidate to explain these observations, the centrosomal associated protein speriolin which has been reported in human and mouse sperm [Goto et al., 2010], however, the mechanism responsible for this rescue remains unclear. The strongest evidence for a paternal donation in our study is the ability of androgenotes to overcome one-cell block when treated under similar conditions as parthenogenotes (Fig. 1).

POTENTIAL RELATIONSHIP BETWEEN CHX INDUCED PARTHENOGENETIC METAPHASE STALL AND ASYMMETRIC ORC2 LEVELS OF PPN VERSUS MPN

We identified two phenomenon that we report here from studying the dynamics of ORC2 during early embryonic development. While

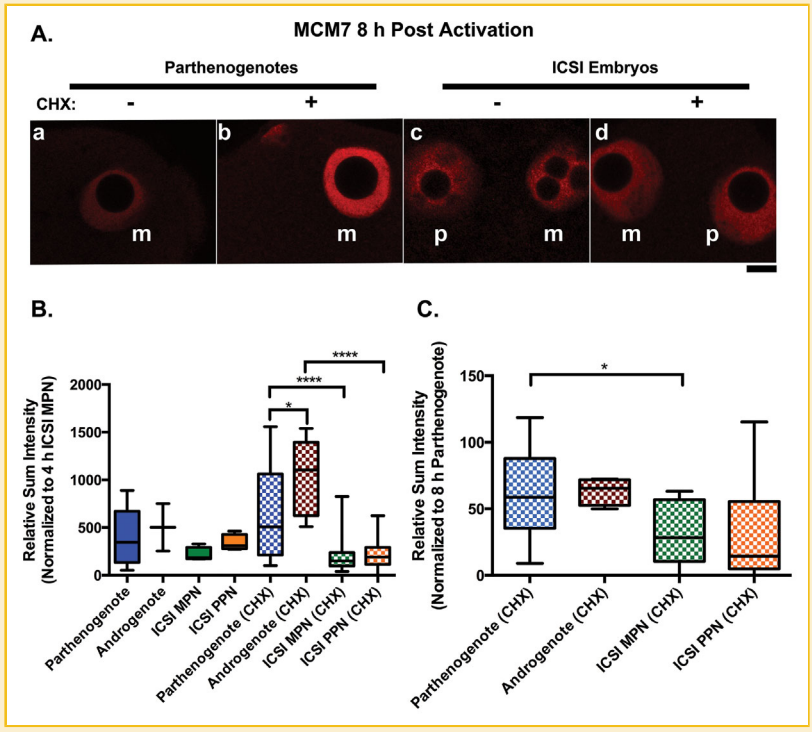

Fig. 3. Effect of CHX on pronuclear MCM7 loading during early zygotic development. (A) After MCM(2–7) are recruited to a potential origin, the site is considered competent for replication. The images represented are from the same co-stained embryos depicted in Figure 2A. All images shown here are single plane 0.25 μm confocal slices. Scale Bar = 5 μm (m = maternal pronucleus, p = paternal pronucleus). (B) Relative MCM7 sum intensity comparisons were normalized to MCM7 at 4 h ICSI in the MPN to show the relative increase after PN formation. From left to right for the groups shown n = 12, 2, 4, 4, 32, 3, 49, 49. (C) The cells analyzed here were from the same treatments as Figure 2C. From left to right for the groups shown n = 17, 4, 17, 17. The number of androgenotes was difficult to quantify (see comment in Fig. 2). For B and C, significance was measured by one-way ANOVA with Tukey post-hoc test, *P* value of <0.05.

the data do not clearly link the asymmetric metaphase block of the CHX treated parthenogenotes with the lower levels of ICC detectable ORC2 in MPN, it is well documented that ORC2 depletion results in metaphase defects in other model systems [Machida et al., 2005; Pflumm and Botchan, 2001], therefore the disruption of the ORC2 observed in parthenogenotes (Fig. 4) suggests this relationship could be true in mammalian embryos as well. However, since other CHX released cells tested here progressed to the 2-cell stage in the presence of paternal DNA when released (Fig. 1) it further suggests that both ORC2 and some paternally donated factor, possibly

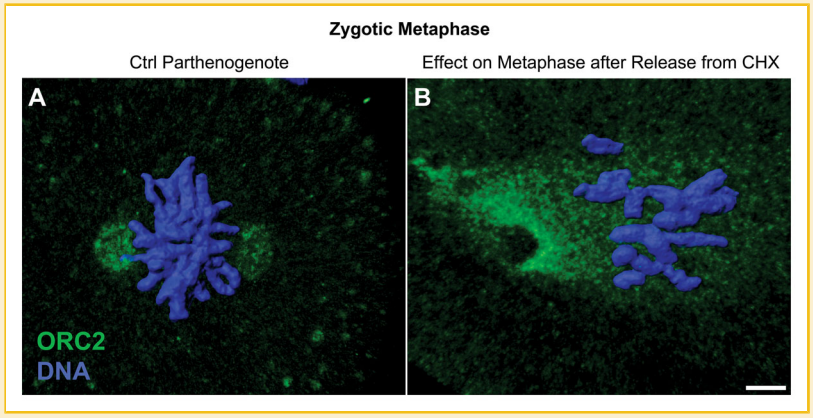

Fig. 4. Parthenogenotes encounter severe metaphase defects after release from prolonged treatment. (A) Control parthenogenotes will enter mitosis (DAPI DNA signals were rendered with Imaris software, blue) with after roughly 18 h. At this point they form metaphase chromatin and ORC2 (green) is evicted to the polar spindle regions. (B) However, parthenogenotes released from CHX after 8 h of continuous treatment encounter severe zygotic metaphase abnormalities. Whenever there appeared to be misaligned ORC2 localization at the spindle poles there was correlatively abnormal metaphase activity as well. Image shown as assembled Z-stack series to emphasis the placement polar spindle placement of ORC2 in relation to metaphase DNA. Scale Bar = 7 μm.

centrosomal material, are required for zygotic division. This is a likely interpretation as non-replicative roles in segregation have previously been linked to other ORC subunits [Chesnokov et al., 2003; Hemerly et al., 2009]. Here, it should be emphasized that while ORC2 displayed dynamic behavior on the MPN when compared to the PPN under CHX treatment, the overall ability to license DNA in the zygote, as detected by salt resistant MCM7, was relatively the same for most chromatin types tested (Fig. 3C).

Taken together, these observations suggest that ORC2 displays chromatin specific dynamic behavior as the data presented here indicates that ORC2 uniquely associates with the maternal and paternal PN. However, what is most clear from these data is that sperm appears to donate translation-independent factors that assist the zygote in division.

AUTHORS' CONTRIBUTIONS

Research design: Michael A. Ortega and W. Steven Ward. Micromanipulation: Joel Marh and Myungjun Ko. Conducted experiments: Michael A. Ortega, Ariel Finberg, Marissa Oshiro. Performed data analysis: Michael A. Ortega. Contributed to writing: Michael A. Ortega and W. Steven Ward.

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