Received: 6 July 2018 | Accepted: 10 September 2018

DOI: 10.1002/jcp.27510

ORIGINAL RESEARCH ARTICLE

WILEY Journal of Cellular Physiology

Intersectin-Cdc42 interaction is required for orderly meiosis in porcine oocytes

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 31771660

Abstract

Intersectins (ITSNs) have been shown to act as adaptor proteins that govern multiple cellular events via regulating Cdc42 activity. However, it remains to be determined whether the ITSN-Cdc42 pathway is functional in porcine oocytes. To address this question, we used a small molecule, ZCL278, to selectively disrupt the ITSN2-Cdc42 interaction. In the present study, we find that porcine oocytes exposed to ZCL278 are unable to completely progress through meiosis. Meanwhile, the spindle defects and chromosomal congression failure are frequently detected in these oocytes. In support of this, we observed the accumulated distribution of vesicle-like ITSN2 signals around the chromosome/spindle region during porcine oocyte maturation. In addition, our results also showed that inhibition of the ITSN-Cdc42 interaction impairs the actin polymerization in porcine oocytes. In summary, the findings support a model where ITSNs, through the interaction with Cdc42, modulates the assembly of meiotic apparatus and actin polymerization, consequently ensuring the orderly meiotic progression during porcine oocyte maturation.

KEYWORDS
Cdc42, intersectin (ITSN), meiosis, oocyte, reproduction

1 | INTRODUCTION

Oocyte quality is a critical element dictating the fertility of a female. In mammals, the full-grown oocyte is the largest cells in the body. Oocytes undergo the complicated reductional division progression during maturation. In the first meiotic prophase, oocytes are arrested at the diplotene stage, which is also known as the germinal vesicle (GV) stage. After stimulation by pituitary luteinizing hormone, oocytes reinitiate meiosis, which is indicated by GV breakdown. At metaphase I (MI) stage, microtubules organize into the specialized bipolar spindle and all chromosomes aligned at the equatorial plate. The chromosome-spindle complex migrates toward one side of the cortex, extruding the first polar body (Pb1). Then oocytes arrested at MII until fertilization activates it to complete the second meiotic division (Clarke, 2017; Meng et al., 2016; Q. C. Wang et al., 2014). In mouse oocytes, actin filaments interleaved with spindle microtubules, ensuring faithful chromosome segregation (Yamagishi & Abe, 2017). Misaligned chromosomes during oocytes meiosis will result in aneuploidy that can be the major genetic causes of miscarriage and infertility. The redistribution of organelles, microtubules, actin filaments, and other cytoskeleton-related proteins provides the framework for these diverse cellular processes (Chen, McMillan-Ward, Kong, Israels, & Gibson, 2007).

Adaptor proteins, also known as scaffold proteins, serve as platforms for the assembly of multiple protein signaling complexes, mediating the interaction between receptors and signal transduction pathways (Tsyba et al., 2011). Adaptor proteins play a pivotal role in diverse cellular processes including cell proliferation, differentiation, and survival control (Brookes, Levonen, Shiva, Sarti, & Darley-Usmar, 2002; Jeganathan, Predescu, & Predescu, 2017). Intersectins (ITSNs) is an evolutionarily conserved adaptor protein family, presenting in diverse metazoan organisms ranging from nematodes to mammals (Tsyba et al., 2011). ITSNs have been reported to engage in endo- and exocytosis, signal transduction, and cytoskeleton rearrangement (Tsyba et al., 2011; L. Zhang et al., 2015). In human, there are two ITSN genes, ITSN1 and ITSN2 are respectively located on chromosomes 21 (q22.1–q22.2) and 2

(pter–p25.1; Guipponi et al., 1998; Pucharcos, Casas, Nadal, Estivill, & de la Luna, 2001). Two isoforms share the same domain organization and are widely expressed in multiple tissues (Aravamudhan, Goldfarb, & Joglekar, 2015; Giménez-Abián et al., 2005; Snetkov, Weisswange, Pfanzelter, Humphries, & Way, 2016). Emerging evidence suggests that ITSN1/2 participates in the cytoskeleton remodeling during mitosis (Bardita, Predescu, Justice, Petrache, & Predescu, 2013; Dergai et al., 2010; Ding et al., 2012; Jakob et al., 2017; Jeganathan et al., 2017; Morderer et al., 2012; Novokhatska et al., 2011). Specifically, expression of DH-PH region in ITSN2 induces the specific activation of Cdc42, leading to the enhanced cortical actin (Klein et al., 2009). Of note, we recently found that ITSN2 controls actin cap formation and meiotic division in mouse oocytes through the Cdc42 pathway (J. Zhang et al., 2017). However, to date, the roles of ITSNs in porcine oocytes have not been addressed yet.

In the present study, by employing a small molecule (ZCL278) targeting Intersectin-Cdc42 interaction, we examined the potential function of ITSNs during porcine oocyte maturation. Our data showed that ZCL278-treated porcine oocytes failed to complete the meiotic maturation, displaying the defects in cytoskeleton organization.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Animals were used and cared according to the Animal Research Institute Committee guidelines prescribed by Nanjing Agricultural University in China. Porcine ovaries were obtained from 6-month-old Duroc Gilts at Nanjing Tianhuan Food Corporation slaughterhouse in China. Ovaries were transported to the laboratory at 25°C in Dulbecco's phosphate-buffered saline (DPBS).

2.2 | Antibodies and chemicals

All chemicals used in this study were purchased from Sigma Chemical Company. Rabbit polyclonal anti-ITSN2 antibody was purchased from Bioss (Beijing, China; bs-13646R). Mouse monoclonal anti-α-tubulin antibodies (A5441), fluorescein isothiocyanate (FITC)-conjugated phalloidin, and FITC-conjugated anti-α-tubulin antibodies (F2168) were purchased from Sigma. FITC-conjugated goat anti-rabbit Immunoglobulin G (IgG) was purchased from ZSGB-BIO (ZF-0311).

2.3 | Porcine oocyte collection and maturation

Porcine ovaries from pubertal gilts were collected at a local slaughterhouse, and then were transferred to the laboratory at 25°C in DPBS containing 500 IU/ml of penicillin/streptomycin. Cumulus-oocyte complexes (COCs) were isolated from medium-sized follicles of ovaries with a 20-gauge needle attached to the syringe. COCs were washed three times in maturation medium: TCM-199 supplemented with 0.1% polyvinyl alcohol, 3.05 mM glucose, 75 mg/L of penicillin, 0.57 mM cysteine, 50 mg/L of streptomycin, 0.91 mM sodium pyruvate, 10% (v/v) pig follicular fluid, 10 ng/ml epidermal growth factor, 10 IU PMSG/ml, and 10 IU hCG/ml. For oocyte in vitro maturation, 80 COCs were transferred to a mircodrop of 500 μl maturation medium and then covered with 200 μl paraffin oil for 44 hr at 38.5°C in a humidified atmosphere of 5% CO_2. For inhibitor treatment, ZCL278 (Cat#: S7293; Selleckchem, Houston, TX) was dissolved in dimethyl sulfoxide and then diluted with maturation medium to the final concentrations of 100, 150, and 200 μM, respectively.

2.4 | Immunofluorescence and confocal microscopy

Denuded porcine oocytes were fixed in 4% paraformaldehyde for 1 hr at room temperature. After multiple washes in phosphate-buffered saline (PBS), oocytes were processed in permeabilization solution (1% Triton X-100, 3 mM $MgCl_2$, 300 mM sucrose, 20 mM HEPES, and 50 mM NaCl in PBS) overnight, and then incubated in 1% BSA in PBS for 1 hr at room temperature. Followed by the incubation with an appropriate secondary antibody, oocytes were labeled with FITC-conjugated Phalloidin, anti-ITSN2 antibodies (1:100), or FITC-conjugated anti-α-tubulin antibodies (1:300). For 24 hr at 4°C. Chromosomes were stained with propidium iodide (PI) for 10 min. Samples were mounted on glass slides within the antifade medium (Vectashield, Burlingame, CA), and then examined under a laser scanning confocal microscope (LSM 700; Zeiss, Oberkochen, Germany).

2.5 | Statistical analysis

At least three independent experiments were repeated for each group, and the results are expressed as mean ± *SD*. Differences between two groups were analyzed by Student's *t* test. Multiple comparisons between more than two groups were analyzed by one-way analysis of variance using Prism 5.0. The $p < 0.05$ was considered to be significant.

3 | RESULTS

3.1 | Subcellular localization of ITSN2 in porcine oocytes

We previously showed that the level of ITSN1 messenger RNA (mRNA) was hardly detected, while ITSN2 mRNA was abundant in oocytes (J. Zhang et al., 2017). We therefore first examined the subcellular localization and expression of ITSN2 during porcine oocyte maturation by immunostaining. Denuded oocytes at GV, Pre-Meta I, MI stage were labeled with anti-ITSN2 antibody and counterstained with PI to visualize DNA. Confocal microscopy revealed that the vesicle-like signals were distributed in the entire cytoplasm at the GV stage. Accompanying the meiotic resumption, ITSN2 signals become concentrated around the chromosome/spindle region (Figure 1), indicative the functional involvement in meiotic regulation.

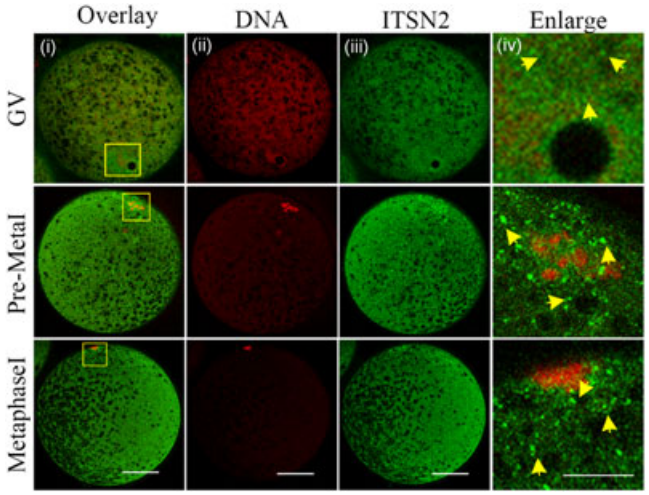

FIGURE 1 Cellular localization of ITSN2 in porcine oocytes. Porcine oocytes at GV, pre-metaphase, and metaphase I stage were immunolabeled with the ITSN2 antibody (green) and counterstained with PI to visualize DNA (red). Arrows indicate the vesicle-like signal of ITSN2. Scale bars, 50 μm. GV: germinal vesicle; ITSN: intersectins; PI: propidium iodide [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | ZCL278 treatment disrupt the maturational progression in porcine oocytes

To check whether Intersectin-Cdc42 interaction is functional in porcine oocytes, ZCL278, a small molecule that specifically targets Cdc42-ITSN interaction and inhibits Cdc42-mediated cellular processes (Friesland et al., 2013), was used in the following experiments. The porcine COCs were cultured in maturation medium supplemented with different concentrations (100, 150, and 200 μM) of ZCL278 for 44 hr (J. Zhang et al., 2017), and then the COCs morphology and maturation progression were evaluated. As shown in Figure 2a, in comparison with control oocytes, the degree of cumulus expansion of ZCL278-treated COCs was significantly lowered (Figure 2ai-iii,, white arrows). Cumulus expansion, an indicator of oocyte maturation, is important for ovulation and fertilization (Seto & Yoshida, 2014). Furthermore, the majority of control porcine oocytes extruded the Pb1 after maturation, whereas the rate of Pb1 emission was decreased in a dose-dependent manner in oocytes exposed to ZCL278 (Figure 2a-[iv–vi] and 2b, red arrows). Two hundred micrometer of ZCL278 was used in the subsequent experiments to disrupt Intersectin-Cdc42 interaction in porcine oocytes.

3.3 | ZCL278 treatment induces the disorganized spindle and misaligned chromosomes in porcine oocyte

Next, we decided to check whether disruption of Intersectin-Cdc42 interaction would alter the meiotic structure in porcine oocytes. For this purpose, control and ZCL278-treated oocytes were immunolabeled with anti-tubulin antibody to observe spindle and co-stained with PI for chromosomes. Confocal microscopy and quantitative

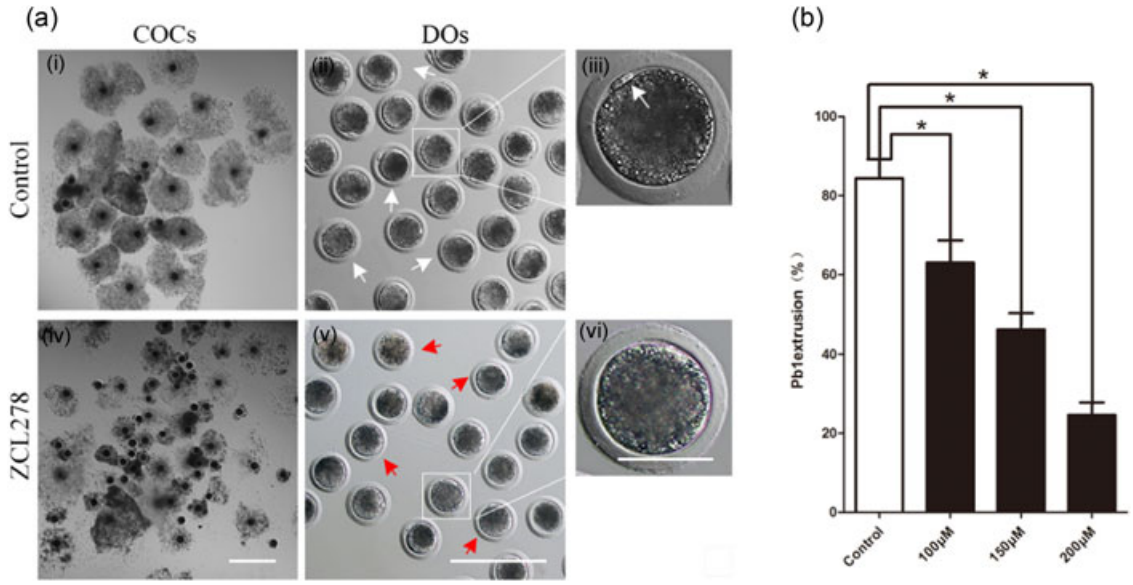

FIGURE 2 Effect of ZCL278 treatment on the meiotic progression of porcine oocytes. (a) Representative DIC images of control and ZCL278-treated (200 μM) oocytes. The oocytes with first polar bodies are indicated by white arrows, and those oocytes without polar bodies are denoted by red arrows. Scale bars, 100 μm. (b) Quantitative analysis of Pb1 extrusion rate in control and ZCL278-treated oocytes. The graph shows the mean ± *SD* of the results obtained in three independent experiments. *$p < 0.05$ versus control. COCs (control, $n = 102$; 200 μM, $n = 118$), DOs (control, $n = 146$; 200 μM, $n = 123$), Pb1 extrusion (control $n = 106$, 100 μM $n = 112$, 150 μM $n = 146$, 200 μM $n = 141$). COCs: cumulus-oocyte complexes; DOs: denuded oocytes; Pb1: first polar body; *SD*: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

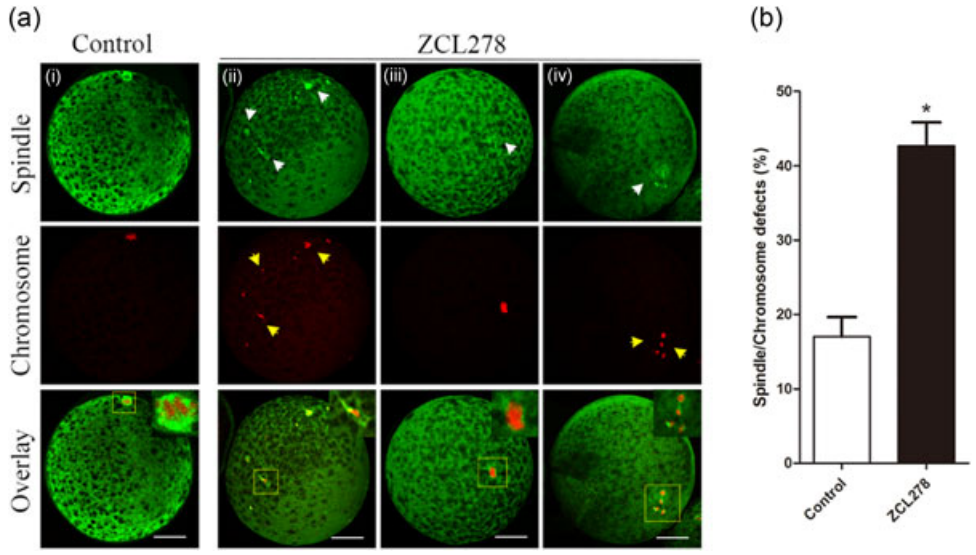

FIGURE 3 Effects of ZCL278 treatment on the meiotic apparatus in porcine oocyte. Oocytes at metaphase II stage were immunostained with anti-tubulin antibody to observe spindles (green) and counterstained with PI for chromosomes (red). (a) Representative confocal images of control and ZCL278-treated oocytes. Abnormal spindles are denoted by white arrows and abnormal chromosomes are indicated by yellow arrows. The details of spindle/chromosomes have been enlarged at the top-right corner of the overlay figure. Scale bars, 20 μm. (b) Quantification of control (n = 116) and ZCL278-treated (n = 107) with spindle/chromosome defects. The graph shows the mean ± *SD* of the results obtained in three independent experiments. $^*p < 0.05$ versus controls. PI: propidium iodide; *SD*: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

analysis (Figure 3a-i and 3b]) revealed that most control oocytes at metaphase stage displayed a typical bipolar spindle with a well-aligned chromosome on the equatorial plate. By contrast, ZCL278-treated oocytes show a higher incidence of spindle defects and chromosome misalignment (Figure 3a-[ii–iv] and 3b; arrows) than that in controls. Altogether, these results suggest that the accurate assembly of meiotic apparatus in porcine oocytes requires Intersectin-Cdc42 interaction.

3.4 | Disruption of Intersectin-Cdc42 interaction influences the actin polymerization in porcine oocytes

To check whether the Intersectin-Cdc42 interaction is essential for the actin polymerization in porcine oocytes, matured control, and ZCL278-treated oocytes were loaded with actin tracker phalloidin and co-stained with PI. As shown in Figure 4a-i, the intact distribution of polymerized actin can be clearly observed on the membrane of normal metaphase oocytes (arrows). Of note, the quantitative analysis demonstrated that ZCL278 treatment impairs the microfilament polymerization during porcine oocyte meiosis (Figure 4a-[ii–iii] and 4b, arrows), indicating that it might be a major factor contributing to the meiotic defects we mentioned above.

4 | DISCUSSION

ITSN is an evolutionarily conserved protein family with a multiple function structure domain (Tsyba et al., 2011). Cdc42, as a member of the Rho family GTPase, functions as a molecular switch controls cell polarity (Smith et al., 2005). Specific deletion of Cdc42 deficiency impairs the oocyte polarization and cytokinesis (Z. B. Wang et al., 2013). It is worth noting that ITSN2 serves as a guanine nucleotide exchange factor for Cdc42 activity via its DH domain (Klein et al., 2009). On the other hand, DH-PH tandem of ITSNs participates in the control of the developmental phenotype of *Xenopus* embryos by modulating cytoskeleton dynamics (Novokhatska et al., 2011). ZCL278 is specifically designed to disrupt the Cdc42-ITSN interaction in cells (J. Zhang et al., 2017). In the present study, we found that ZCL278 treatment not only inhibited the expansion of cumulus cells but also induced the meiotic arrest (Figure 2). In particular, the deficient spindle formation and chromosome organization were readily detected in porcine oocytes exposed ZCL278 (Figure 3). This finding strongly suggests that the interaction between ITSN and Cdc42 plays an important role in the assembly of meiotic structure in porcine oocytes. In support of this conclusion, we observed the accumulated distribution of vesicle-like ITSN2 signals around the chromosome/spindle region during porcine oocyte maturation (Figure 1). Similarly, specific depletion of ITSN2 results in the delayed meiotic resumption and defective cytokinesis upon in mouse oocytes, displaying the disorganized cytoskeleton (J. Zhang et al., 2017).

Before fertilization, mammalian oocyte undergoes an asymmetric division, producing a small Pb and a big functional haploid egg. Due to the absence of centriole, the asymmetric division depends on the subcortical spindle positioning and establishment of a cortical actomyosin domain overlying the spindle (Guo et al., 2015; Xie et al., 2018; Yi et al., 2013). Actin filaments are well known for their significant roles in spindle migration and anchorage to the cortex in meiotic oocytes (Metchat et al., 2015; Yamagishi & Abe, 2015).

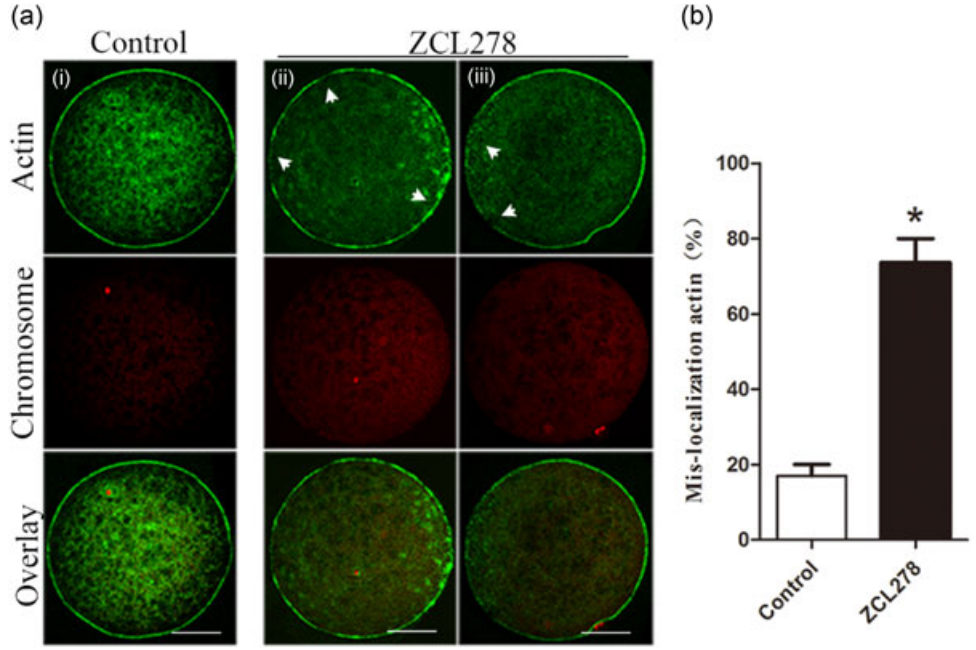

FIGURE 4 Effects of ZCL278 treatment on the actin polymerization in porcine oocyte. MII oocytes were labeled with phalloidin to visualize actin (green) and counterstained with PI for chromosomes (red). (a) Representative confocal images show actin distribution in control (i) and ZCL278-treated (ii,iii) oocytes. Arrows indicate the defective polymerization of actin. Scale bars, 20 μm. (b) Quantitative analysis of abnormal actin distribution in control ($n = 47$) and ZCL278-treated oocytes ($n = 52$). Each group and data are expressed as means ± *SD*. $^{*}p < 0.05$ versus controls. MII: metaphase II; *SD*: standard deviation [Color figure can be viewed at wileyonlinelibrary.com]

Defects in F-actin polymerization lead to the failure of Pb extrusion during oocyte maturation (Somfai et al., 2011). Cdc42 cycles between a GTP-bound active and a GDP-bound inactive conformation (Smith et al., 2005). Activated Cdc42 is concentrated in the cortical region overlying meiotic chromosomes (Dehapiot, Carrière, Carroll, & Halet, 2013). Furthermore, actin cap formation and cytokinesis were disturbed in mouse oocytes expressing the dominant-negative mutant of Cdc42 (Clarke, 2017). Importantly, our results also show that inhibition of the ITSN-Cdc42 interaction influences the actin polymerization in porcine oocytes (Figure 4). The downstream effector proteins of Cdc42, such as WASP and Arp2, have been suggested to mediate these phenotypes in mouse oocytes (J. Zhang et al., 2017). Collectively, our data support a model where ITSN2, through the interaction with Cdc42, modulates the assembly of meiotic apparatus and polymerization of the actin cytoskeleton, consequently ensuring the orderly meiotic progression during porcine oocyte maturation.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (No. 31771660).

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

X.L. and L.G. designed the research. X.L., X.L., M.G., and Y.H. performed the research. X.L., B.X., H.L., and L.G. analyzed the data. X.L. and L.G. wrote the paper.

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How to cite this article: Li X, Gao M, He Y, Xiong B, Liu H, Gu L. Intersectin-Cdc42 interaction is required for orderly meiosis in porcine oocytes. *J Cell Physiol*. 2018;1–6. https://doi.org/10.1002/jcp.27510