

# Ca<sup>2+</sup> influx and the store-operated Ca<sup>2+</sup> entry pathway undergo regulation during mouse oocyte maturation

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**ABSTRACT** In preparation for fertilization, mammalian oocytes undergo optimization of the mechanisms that regulate calcium homeostasis. Among these changes is the increase in the content of the Ca<sup>2+</sup> stores ([Ca<sup>2+</sup>]<sub>ER</sub>), a process that requires Ca<sup>2+</sup> influx. Nevertheless, the mechanism(s) that mediates this influx remains obscure, although it is known that [Ca<sup>2+</sup>]<sub>ER</sub> can regulate Ca<sup>2+</sup> influx via store-operated Ca<sup>2+</sup> entry (SOCE). We find that during maturation, as [Ca<sup>2+</sup>]<sub>ER</sub> increases, Ca<sup>2+</sup> influx decreases. We demonstrate that mouse oocytes/eggs express the two molecular components of SOCE—stromal interaction molecule 1 (Stim1) and Orai1—and expression of human (h) Stim1 increases Ca<sup>2+</sup> influx in a manner that recapitulates endogenous SOCE. We observe that the cellular distribution of hStim1 and hOrai1 during maturation undergoes sweeping changes that curtail their colocalization during the later stages of maturation. Coexpression of hStim1 and hOrai1 enhances influx throughout maturation but increases basal Ca<sup>2+</sup> levels only in GV oocytes. Further, expression of a constitutive active form of hStim1 plus Orai1, which increases basal Ca<sup>2+</sup> throughout maturation, disturbs resumption of meiosis. Taken together, our results demonstrate that Ca<sup>2+</sup> influx and SOCE are regulated during maturation and that alteration of Ca<sup>2+</sup> homeostasis undermines maturation in mouse oocytes.

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## INTRODUCTION

Changes in the intracellular concentration of free calcium ([Ca<sup>2+</sup>]<sub>i</sub>) represent an important signaling mechanism involved in a wide

range of cellular events, including muscle contraction, secretion, neurotransmission, and cell death (Berridge *et al.*, 2000). [Ca<sup>2+</sup>]<sub>i</sub> signaling also plays a dominant role during fertilization in all species examined (Stricker, 1999; Runft *et al.*, 2002; Malcuit *et al.*, 2006; Swann *et al.*, 2006). In mammals, the sperm-induced [Ca<sup>2+</sup>]<sub>i</sub> signal adopts a pattern of brief [Ca<sup>2+</sup>]<sub>i</sub> rises interspersed among long intervals of basal concentration, which are referred to as [Ca<sup>2+</sup>]<sub>i</sub> oscillations. The oscillations are believed to be initiated by a sperm-specific phospholipase C (PLC), PLC zeta 1 (ζ), after fusion of the gametes (Saunders *et al.*, 2002). PLCζ is believed to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), resulting in the production of 1,4,5-inositol-trisphosphate (IP<sub>3</sub>), the ligand for IP<sub>3</sub>R1, the Ca<sup>2+</sup> channel located in the endoplasmic reticulum (ER), the egg's main Ca<sup>2+</sup> store (Miyazaki *et al.*, 1992; Miyazaki, 2006; Ducibella and Fissore, 2008). Activation of this pathway causes the initial intracellular Ca<sup>2+</sup> release, but persistence of the oscillations requires Ca<sup>2+</sup> influx, as without external Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>e</sub>) only a few rises occur after sperm entry (Kline and Kline, 1992). Notwithstanding the importance of Ca<sup>2+</sup> influx, the mechanisms that underlie it during mammalian fertilization are unknown.

Fertilization in most vertebrate species happens at the metaphase II stage of meiosis (MII), although changes in Ca<sup>2+</sup> homeostasis that

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Abbreviations used: AP, antigenic peptide; BSA, bovine serum albumin; CAD, CRAC-activating domain; [Ca<sup>2+</sup>]<sub>e</sub>, external Ca<sup>2+</sup>; [Ca<sup>2+</sup>]<sub>ER</sub>, Ca<sup>2+</sup> in endoplasmic reticulum; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular concentration of free calcium; CIP, calf intestine phosphatase; CRAC, Ca<sup>2+</sup> release-activated Ca<sup>2+</sup>; cRNA, complementary RNA; CZB, Chatot, Ziomek, and Bavister medium; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; Fura-2AM, Fura-2 acetoxymethyl ester; GV, germinal vesicle; GVBD, germinal vesicle breakdown; HCZB, HEPES-buffered CZB; IBMX, 3-isobutyl-1-methylxanthine; IP<sub>3</sub>, 1,4,5-inositol-trisphosphate; IP<sub>3</sub>R1, 1,4,5-inositol-trisphosphate receptor 1; MI, metaphase I; MII, metaphase II; mRFP, modified red fluorescent protein; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; PMSG, pregnant mare serum gonadotropin; PVA, polyvinyl alcohol; SEM, standard error of mean; SERCA, sarcoendoplasmic reticulum Ca<sup>2+</sup>-ATPase; SOCE, store-operated Ca<sup>2+</sup> entry; Stim1, stromal interaction molecule 1; TG, thapsigargin; TL-HEPES, Tyrode's lactate solution; WGA, wheat germ agglutinin; YFP, yellow fluorescent protein.

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occur before this stage during maturation enable eggs to mount  $[Ca^{2+}]_i$  oscillations. Fully grown mammalian oocytes are arrested at the germinal vesicle stage (GV) and are endowed with  $Ca^{2+}$  stores ( $[Ca^{2+}]_{ER}$ ) low in  $Ca^{2+}$  reserves. As maturation ensues after the luteinizing hormone surge,  $[Ca^{2+}]_{ER}$  increases steadily until the MII stage (Tombes *et al.*, 1992; Jones *et al.*, 1995), enhancing IP<sub>3</sub>R1-mediated  $Ca^{2+}$  release and promoting the acquisition of fertilization-like oscillations (Miyazaki *et al.*, 1993; Mehlmann and Kline, 1994). The mechanism(s) that underlie this increase in  $[Ca^{2+}]_{ER}$  and the plasma membrane (PM) channels that underpin the  $Ca^{2+}$  influx are unknown, although insights may be gleaned from the spontaneous  $[Ca^{2+}]_i$  oscillations displayed by GV oocytes (Carroll and Swann, 1992; Carroll *et al.*, 1994). These oscillations require  $Ca^{2+}$  influx and end approximately as the resumption of meiosis begins (Carroll and Swann, 1992), which is when the first increase in  $[Ca^{2+}]_{ER}$  content is noted (Jones *et al.*, 1995). These results both predict an association between  $[Ca^{2+}]_{ER}$  content and  $Ca^{2+}$  influx, which is reminiscent of the mechanism believed to underlie store-operated  $Ca^{2+}$  entry (SOCE; Putney, 1986; Hoth and Penner, 1993) and active regulation of  $Ca^{2+}$  influx during maturation.

SOCE was first proposed as a means for cells to refill  $Ca^{2+}$  stores after  $Ca^{2+}$  release induced by agonist stimulation (Putney, 1986). Subsequent studies showed that depletion of  $[Ca^{2+}]_{ER}$  caused by inhibitors of the sarcoplasmic/endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA) pumps such as thapsigargin (TG) also triggered  $Ca^{2+}$  influx, demonstrating the prevalence of SOCE in somatic cells (Takemura *et al.*, 1989; Hoth and Penner, 1993; Zweifach and Lewis, 1993; Hsu *et al.*, 2001). Subsequent electrophysiological studies revealed unique properties of this current, which was named  $I_{CRAC}$  (Hoth and Penner, 1993; Zweifach and Lewis, 1993; Parekh *et al.*, 1997; Putney, 2009). Although unknown for many years, the molecular effectors of SOCE have been identified, and two components, stromal interaction molecule 1 (Stim1), which acts as the ER  $Ca^{2+}$  sensor (Liou *et al.*, 2005; Roos *et al.*, 2005; Collins and Meyer, 2011), and Orai1, the PM channel that mediates  $Ca^{2+}$  influx upon Stim1-induced oligomerization (Feske *et al.*, 2006; Vig *et al.*, 2006), are believed to coordinate  $Ca^{2+}$  influx after  $Ca^{2+}$  release. The presence of SOCE has been well characterized during the early stages of maturation in *Xenopus* oocytes (Yu *et al.*, 2009), and  $Ca^{2+}$  influx has been described in mammalian eggs during fertilization and after addition of TG (Kline and Kline, 1992; McGuinness *et al.*, 1996; Mohri *et al.*, 2001), although the mechanism(s) and molecules underlying this  $Ca^{2+}$  entry remain to be identified. Consistent with this, detection of transcripts for Stim1 and Orai1 has been reported in mammalian oocytes, although protein expression and cellular distribution during maturation require additional characterization (Gomez-Fernandez *et al.*, 2009, 2012; Koh *et al.*, 2009; Wang *et al.*, 2012).

Although  $Ca^{2+}$  influx is required for  $[Ca^{2+}]_i$  oscillations at the GV stage, its contributions to the filling of  $[Ca^{2+}]_{ER}$  have not been carefully examined. Further, the role of  $[Ca^{2+}]_i$  changes during oocyte maturation is unclear. For instance, in mouse GV oocytes suppression of spontaneous oscillations with the  $Ca^{2+}$  chelator BAPTA-AM did not affect resumption of meiosis, although it caused cell cycle arrest at metaphase I (MI) of meiosis (Tombes *et al.*, 1992). In porcine and bovine oocytes, addition of BAPTA-AM prevented resumption of meiosis, and removal of  $[Ca^{2+}]_e$  precluded progression of meiosis beyond the MI stage (Homa, 1991; Kaufman and Homa, 1993). Remarkably, in *Xenopus* oocytes, in which SOCE is inactivated during maturation (Machaca and Haun, 2000), increased  $Ca^{2+}$  influx by elevation of  $[Ca^{2+}]_e$  and persistent increase in  $[Ca^{2+}]_i$  delayed resumption of meiosis and caused spindle abnormalities (Sun and Machaca, 2004). Whether enhanced  $Ca^{2+}$  influx and persistent

elevation of basal  $[Ca^{2+}]_i$  affect the initiation or progression of maturation in mammalian oocytes has not been examined.

In this study in mouse oocytes we investigate whether  $Ca^{2+}$  influx contributes to the filling of  $[Ca^{2+}]_{ER}$  during maturation and whether it is differentially regulated during this process. We also study whether SOCE contributes to this influx, as well as the expression of the molecular components of SOCE and their regulation during maturation. Finally, we evaluate the impact of persistently elevated  $[Ca^{2+}]_i$  on resumption and progression of meiosis.

## RESULTS

### Spontaneous $Ca^{2+}$ influx is suppressed during maturation in parallel with the increase of ER $Ca^{2+}$ store

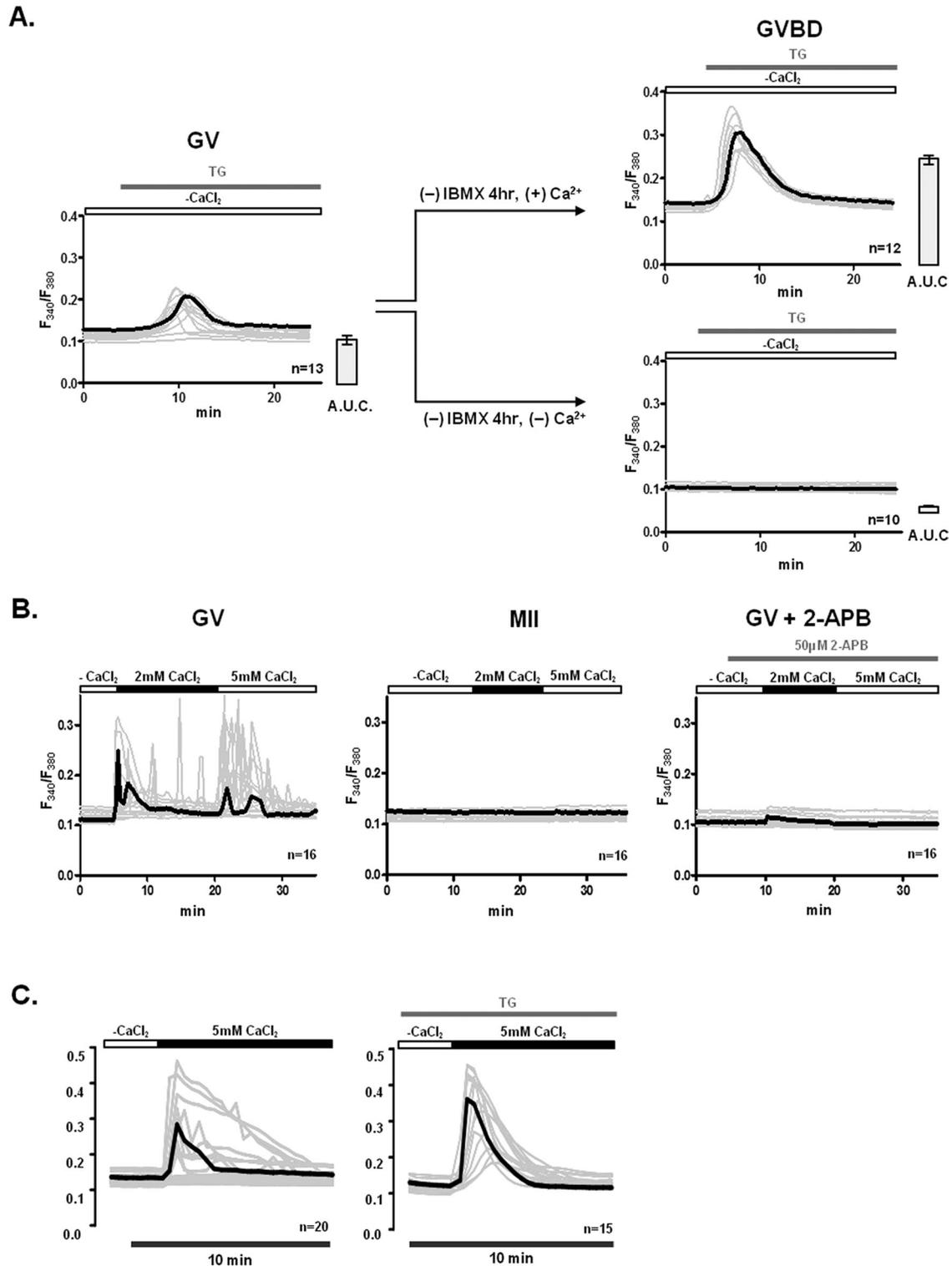
To gain insight into the mechanism and molecular effectors that mediate  $Ca^{2+}$  influx in mouse oocytes and eggs, we ascertained whether  $Ca^{2+}$  influx across the PM was required for the increase in  $[Ca^{2+}]_{ER}$  that occurs during the transition from the GV to the germinal vesicle breakdown (GVBD) stage (Jones *et al.*, 1995). To accomplish this, GV oocytes were allowed to transition to the GVBD stage in the presence/absence of extracellular  $Ca^{2+}$  (1.7 mM). We estimated  $[Ca^{2+}]_{ER}$  levels after addition of TG 4 h after the removal of 3-isobutyl-1-methylxanthine (IBMX). In the presence of extracellular  $Ca^{2+}$ ,  $[Ca^{2+}]_{ER}$  increased significantly in GVBD oocytes (Figure 1A, top, left and right), although this was not the case when maturation was initiated in nominal  $Ca^{2+}$ -free medium (Figure 1A, bottom, right). These results therefore demonstrate that  $[Ca^{2+}]_e$  is required for  $[Ca^{2+}]_{ER}$  increase during maturation.

We next examined whether the  $Ca^{2+}$  entry pathway(s) that mediate  $Ca^{2+}$  influx in GV oocytes are also functional in MII eggs, as both of these cellular stages are known to support oscillations. We incubated GV oocytes and MII eggs in nominal  $Ca^{2+}$ -free media and soon thereafter sequentially added 2 and 5 mM  $CaCl_2$  while we monitored  $[Ca^{2+}]_i$  responses. Although most GV oocytes responded to the addition of  $CaCl_2$  by displaying a noticeable  $[Ca^{2+}]_i$  rise and in some cases oscillations (Figure 1B, left), MII eggs displayed no such changes (Figure 1B, middle). We next examined whether the  $Ca^{2+}$  entry at the GV stage could be abrogated by pretreatment with 50  $\mu$ M 2-APB, a pharmacological agent that has been shown to regulate SOCE (DeHaven *et al.*, 2008); addition of 2-APB prevented the increase in  $[Ca^{2+}]_i$  after the addition of either concentration of  $CaCl_2$  (Figure 1B, right).

The inhibitory effect of 2-APB and the low  $[Ca^{2+}]_{ER}$  levels in GV oocytes suggested that SOCE may be involved, at least in part, in mediating  $Ca^{2+}$  entry in GV oocytes. Accordingly, we investigated whether  $Ca^{2+}$  influx at the GV stage could be modified by exposure to TG before  $CaCl_2$  add back, a method commonly used to test SOCE in somatic cells (Bird *et al.*, 2008; DeHaven *et al.*, 2008). Although 3 of 20 untreated oocytes showed large and prolonged  $[Ca^{2+}]_i$  responses after addition of  $CaCl_2$ , the majority of oocytes, 9 and 8 of 20, respectively, showed moderate or minor responses (Figure 1C, left). Conversely, TG-exposed oocytes uniformly responded to the addition of  $CaCl_2$  with robust  $[Ca^{2+}]_i$  responses (Figure 1C, right). Taken together, these results show that  $Ca^{2+}$  entry is functionally regulated during mouse oocyte maturation and that SOCE is one of the underlying mechanisms of  $Ca^{2+}$  influx at the GV stage.

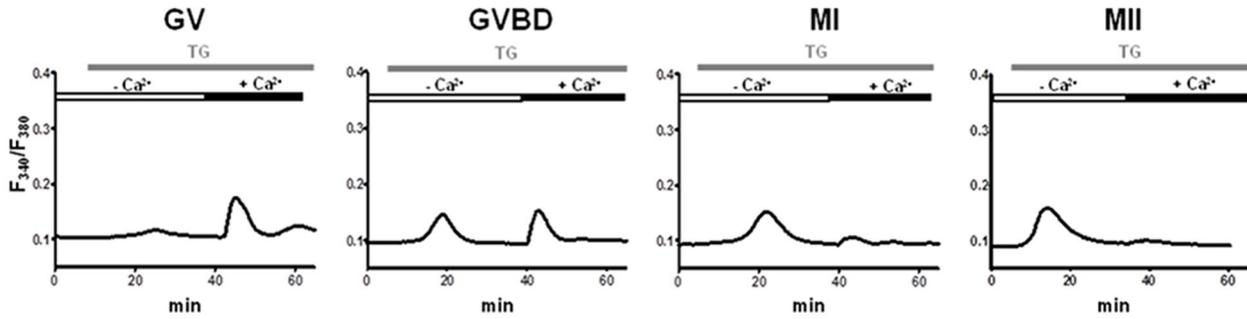
### $[Ca^{2+}]_{ER}$ content and $Ca^{2+}$ influx undergo distinct regulation during mouse oocyte maturation

The previous findings led us to investigate the function of SOCE throughout oocyte maturation, using the TG and  $CaCl_2$  add-back method. Addition of TG, as expected, caused an increase in  $[Ca^{2+}]_i$  that became gradually higher as maturation progressed (Figure 2, A and C;  $p < 0.05$ ), which is in agreement with previous

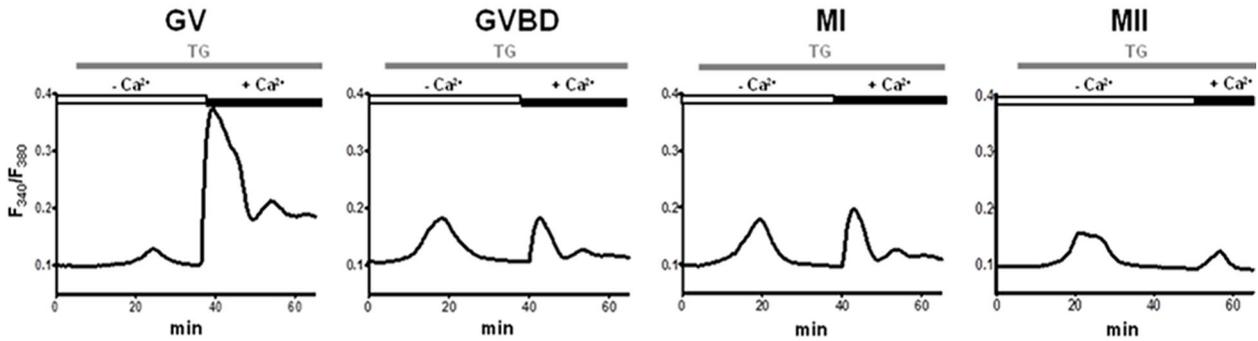


**FIGURE 1:**  $[Ca^{2+}]_e$  and  $Ca^{2+}$  influx are required to fill  $[Ca^{2+}]_{ER}$  in oocytes. The underlying  $Ca^{2+}$  influx mechanism(s) are inactivated during maturation and are sensitive to 2-APB and TG at the GV stage. (A) The contribution of extracellular  $Ca^{2+}$  to  $[Ca^{2+}]_{ER}$  content was estimated in GVBD-stage oocytes after culturing GV oocytes for 4 h in media supplemented with 1.7 mM  $CaCl_2$  or without supplementation, nominal  $Ca^{2+}$ -free medium. Release of  $[Ca^{2+}]_{ER}$  was induced by addition of 10  $\mu$ M TG. All  $[Ca^{2+}]_i$  responses are shown in the graphs, and the bold trace in each graph represents the mean response; bar graphs to the right of each  $Ca^{2+}$  panel denote mean  $\pm$  SEM of  $[Ca^{2+}]_{ER}$  content estimated as area under the curve. (B) Spontaneous  $Ca^{2+}$  influx was measured in GV oocytes and MII eggs. Oocytes and eggs were placed in  $Ca^{2+}$ -free conditions, after which 2 and 5 mM  $CaCl_2$  were successively added. Given that only GV oocytes showed  $Ca^{2+}$  influx, they were pretreated with 50  $\mu$ M 2-APB for 5 min before addition of  $CaCl_2$  to prevent influx. (C)  $Ca^{2+}$  influx was promoted by addition of  $CaCl_2$  into GV oocytes with and without prior treatment with TG. GV oocytes were placed in nominal  $Ca^{2+}$ -free media or exposed to 10  $\mu$ M TG for 30 min in nominal  $Ca^{2+}$ -free medium to deplete  $[Ca^{2+}]_{ER}$ , after which 5 mM  $CaCl_2$  was added. Representative traces are shown, and bold trace represents mean response.

## A. Control

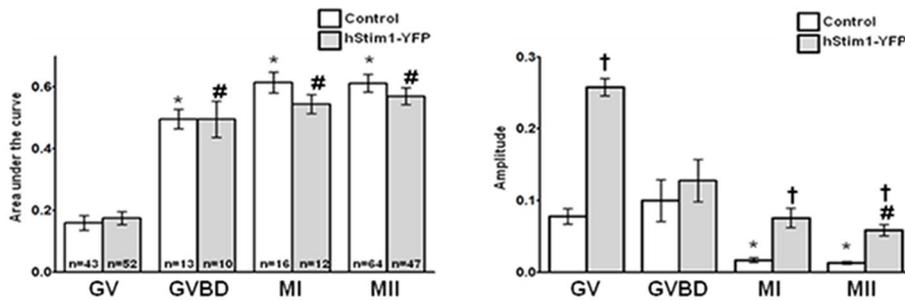


## B. hStim1-YFP overexpression

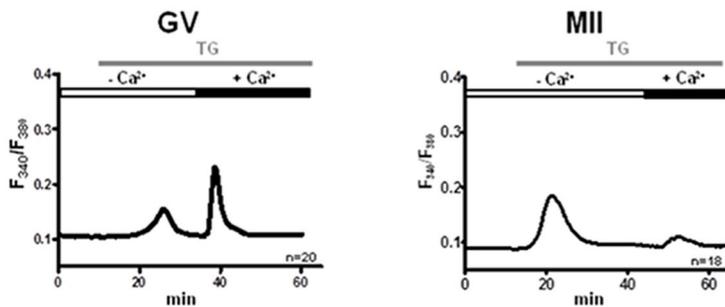


## C. TG-induced Ca<sup>2+</sup> release

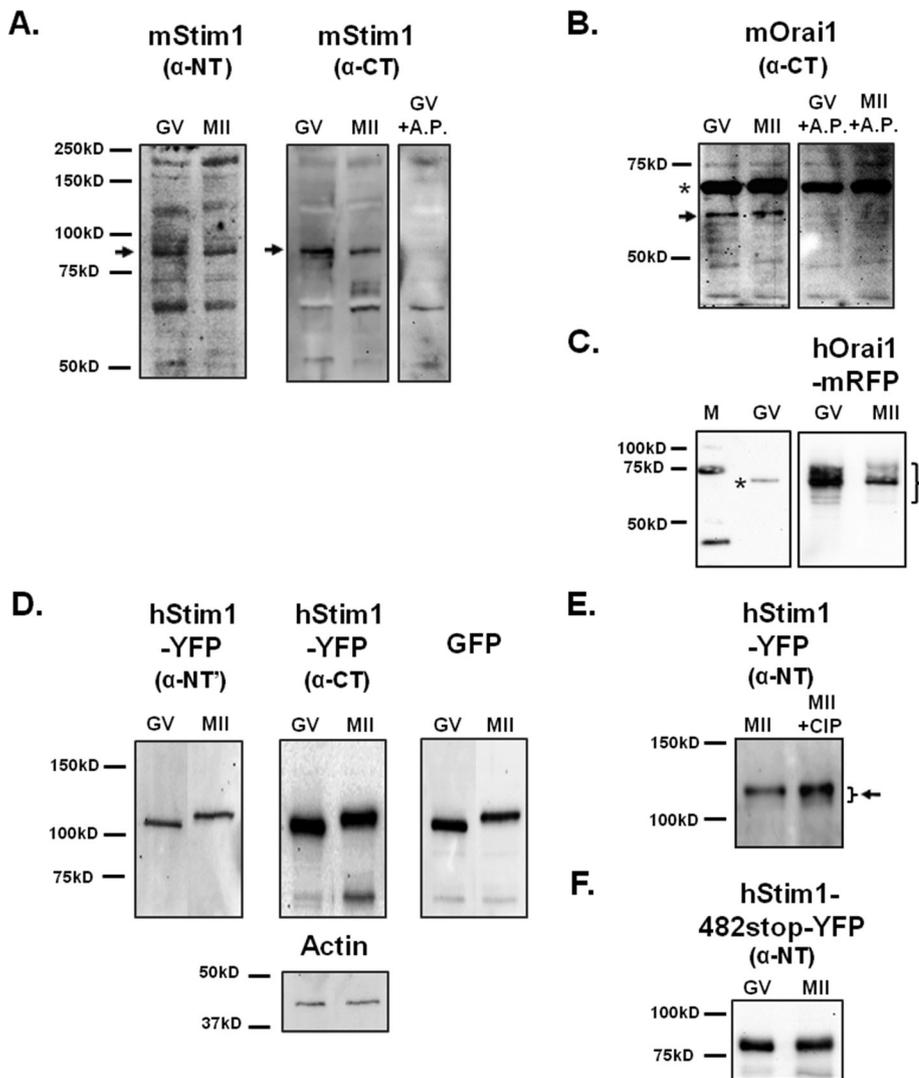
## Ca<sup>2+</sup> influx



## D. hStim1-ΔCAD-YFP overexpression



**FIGURE 2:**  $[Ca^{2+}]_{ER}$  content increases, whereas  $Ca^{2+}$  influx induced by TG, SOCE, decreases during oocyte maturation.  $[Ca^{2+}]_{ER}$  content was estimated from the  $[Ca^{2+}]_i$  responses caused by addition of 10  $\mu M$  TG in oocytes incubated in  $Ca^{2+}$ -free medium, and  $Ca^{2+}$  influx was estimated by the  $[Ca^{2+}]_i$  rise generated by the addition of 5 mM  $CaCl_2$  soon after the TG-induced  $[Ca^{2+}]_i$  rise had subsided. Representative  $[Ca^{2+}]_i$  traces are shown for control oocytes (A) or for oocytes expressing hStim1-YFP (B). (C) TG- and  $CaCl_2$ -induced  $[Ca^{2+}]_i$  changes were quantified, and data are presented in bar graphs as mean  $\pm$  SEM. Control and hStim1-YFP-expressing oocytes are displayed in open and gray columns, respectively, and the number of oocytes evaluated is shown within each bar. \*#Stages significantly different from the GV-stage values within treatment group. †Significant differences within the same meiotic stage but between treatments ( $p < 0.05$ ). (D) TG-induced  $[Ca^{2+}]_i$  responses and subsequent  $Ca^{2+}$  influx in oocytes and eggs expressing hStim1- $\Delta CAD$ -YFP, a Stim1 variant incapable of interacting with Orai1.



**FIGURE 3:** Mouse oocytes express Stim1 and Orai1. (A, B) The expression of Stim1 and Orai1 molecules was probed using lysates of GV oocytes ( $n = 100$  and  $200$ , respectively) and MII ( $n = 100$  and  $200$ , respectively) eggs and specific antibodies. (A) Left and middle, black arrows point to the band corresponding to Stim1. An antigenic peptide was used to confirm the specificity of the anti-C-terminus Stim1 antibody. (B) Right, black arrow points to the band that represents Orai1. The upper band of  $\sim 68$  kDa marked with an asterisk is believed to be nonspecific reactivity, as it was not abolished by pretreatment with the antigenic peptide. (C) hOrai1-mRFP expression ( $n = 16$  GV oocytes/ MII eggs) was analyzed in overexpressing cells using the same antibody. (D) Heterologous expression of hStim1-YFP was demonstrated in lysates of oocytes/eggs ( $n = 45$ ) using the anti-N-terminus and anti-C-terminus Stim1 antibodies and an anti-GFP antibody. The actin protein was probed as a loading control (bottom, middle). (E) To examine the possible phosphorylation of hStim1-YFP in mouse oocytes, lysates of hStim1-YFP-expressing MII oocytes ( $n = 18$ ) were incubated with or without calf intestine phosphatase and then immunoblotted with anti-Stim1 antibody (right). The presence of hStim1 is denoted by an arrow, and the bracket denotes the presence of polypeptides of different MWs. (F) hStim1-482stop-YFP-overexpressing GV and MII cells ( $n = 18$ ) were probed with the anti-N-terminus Stim1 antibody, and blots are shown.

reports (Kline and Kline, 1992; Jones *et al.*, 1995) and suggests increasing  $[Ca^{2+}]_{ER}$  content with progression of maturation. In contrast, after  $CaCl_2$  addition,  $Ca^{2+}$  influx progressively decreased during maturation, with GV- and GVBD-stage oocytes displaying greater  $Ca^{2+}$  influx than MI and MII eggs (Figure 2, A and C;  $p < 0.05$ ), demonstrating gradual inactivation of  $Ca^{2+}$  entry with progression of maturation.

genic peptide specifically obliterated recognition of this band (Figure 3A, right). Expression of Orai1 was also detected in mouse eggs (Figure 3B, left) with an approximate MW of 56 kDa, which, although higher than predicted for the native protein, is consistent with the MW of the protein in some mammalian tissues (Balghi *et al.*, 2011); Orai1 has been shown to be glycosylated in somatic cells (Prakriya *et al.*, 2006; Gwack *et al.*, 2007). Preincubation of the antibody with

The inverse relationship between  $[Ca^{2+}]_{ER}$  content and  $Ca^{2+}$  entry during maturation indicates the participation of SOCE as one of the mechanisms involved in  $Ca^{2+}$  homeostasis in mouse oocytes. Remarkably, our results suggest that SOCE is progressively disabled during maturation. To extend these observations, we injected GV-stage oocytes with hStim1–yellow fluorescent protein (YFP) cRNA, the  $Ca^{2+}$  sensor component of SOCE, and examined the effects of such injections on  $[Ca^{2+}]_{ER}$  and  $Ca^{2+}$  influx during different stages of maturation. hStim1 and mStim1 are highly homologous, sharing  $\sim 97\%$  of the amino acids at the whole-protein level and  $99\%$  of the amino acids in the CRAC-activating domain (CAD), which is the domain that directly interacts with Orai1 (Park *et al.*, 2009). Therefore hStim1-YFP overexpression was expected to mimic mStim1 function. Expression of hStim1-YFP did not affect  $[Ca^{2+}]_{ER}$  content throughout maturation (Figure 2, B and C, left), at least as estimated by our approach, but clearly enhanced  $Ca^{2+}$  influx in all stages of maturation, especially in GV oocytes (Figure 2, B and C, right;  $p < 0.05$ ). To demonstrate that the enhanced influx was due to interaction of hStim1 with endogenous Orai1, we injected oocytes with hStim1- $\Delta$ CAD-YFP mRNA, which cannot activate Orai1 (Park *et al.*, 2009); overexpression of hStim1- $\Delta$ CAD-YFP failed to enhance  $Ca^{2+}$  influx at any stage of maturation (Figure 2D). Collectively the data suggest that hStim1-YFP overexpression broadly recapitulates the regulation of  $[Ca^{2+}]_{ER}$  and  $Ca^{2+}$  influx observed in mouse oocytes, suggesting that SOCE is operational during mouse oocyte maturation, although it is seemingly inactivated at the most advanced stages of meiosis.

### Stim1 and Orai1 are expressed in mouse oocytes

We next examined in mouse oocytes/eggs the expression of the two molecular effectors of SOCE—Stim1 and Orai1. Two different anti-Stim1 antibodies were used for Western blotting, and both antibodies detected a band of  $\sim 90$  kDa in GV and MII stages (Figure 3A, left and center), which is the reported molecular weight (MW) of Stim1 (Darbellay *et al.*, 2011); the observed reactivity was likely Stim1's, as preincubation of the anti-C-terminus antibody with an anti-

its antigenic peptide abrogated the reactivity of the 56 kDa band but not that of a prominent higher, nonspecific band (Figure 3B, right; asterisk), confirming the specificity of the antibody. Further, the same antibody detected expression of hOrai1–monomeric red fluorescent protein (mRFP) in oocytes and eggs, where it detected several polypeptides ranging in MW from ~52 to 82 kDa, likely the reflection of various degrees of glycosylation (Figure 3C).

To confirm the functional results obtained by injection of hStim1-YFP cRNA, we examined the expression of hStim1-YFP in GV and MII oocytes. Both anti-Stim1 antibodies recognized a band at ~105 kDa, which is consistent with the MW of hStim1 and the added MW of YFP (Figure 3D, left and middle); an anti-YFP antibody recognized the same bands (Figure 3D, right). Actin was used as loading control, and Western blotting against it revealed approximate equal loading of the samples (Figure 3D, bottom, middle). In all cases, hStim1-YFP migration was more retarded in MII eggs than in GV oocytes (Figure 3D), suggesting phosphorylation of hStim1-YFP, as reportedly occurs in *Xenopus* oocytes and in mammalian somatic cells (Smyth *et al.*, 2009; Yu *et al.*, 2009). To ascertain whether this was also the case in our system, we injected hStim1-YFP cRNAs into GV oocytes; these cells matured to the MII stage, at which time lysates were prepared and either left untreated or treated with alkaline phosphatase (AP) to induce widespread dephosphorylation. Compared to untreated controls, the AP-treated hStim1-YFP displayed a smeared migration, which suggests different degrees of phosphorylation, and higher reactivity (Figure 3E), which is possibly due to better antibody recognition, as the antibody's epitope falls within this domain. To confirm this observation, we injected hStim1-482stop-YFP cRNA, which encodes for a protein that lacks all the C-term M-phase kinase phosphorylation sites (Smyth *et al.*, 2009); hStim1-482stop did not undergo a mobility shift during maturation (Figure 3F). Collectively our data show that the components of SOCE are expressed in mouse oocytes/eggs and that during maturation hStim1 undergoes phosphorylation.

### **hStim1-YFP and hOrai1-mRFP undergo changes in distribution during oocyte maturation**

Such findings led us to examine whether the decline in  $\text{Ca}^{2+}$  influx during maturation coincided with changes in the cellular distribution of Stim1 and Orai1. To follow their distribution, we injected oocytes with hStim1-YFP or hOrai1-mRFP mRNAs. All oocytes were injected at the GV stage and remained at this stage in media supplemented with IBMX for variable times, so that by 20 h postinjection all stages of maturation could be simultaneously examined. hStim1-YFP underwent marked changes in distribution with progression of meiosis. For example, at the GV stage, hStim1-YFP displayed a "patched" distribution, with these patches spread throughout the cell (Figure 4A). In GVBD oocytes, ~4 h after removal of IBMX, large internal patches were still observed in most oocytes (Figure 4B, top), but in ~30% of the cells the distribution of hStim1 became more diffuse, although some patches remained around the spindle area (Figure 4B, bottom). As maturation progressed, the distribution of hStim1 became more disperse and acquired a pattern consistent with its ER localization (Figure 4, C and D, top), although a small number of MI and MII oocytes still showed internal patches of smaller size (Figure 4, C and D, bottom); ER distribution was confirmed by injection of ds-Red ER cRNA (data not shown).

The distribution of hOrai1-mRFP also changed during maturation (Figure 4, E and F). For example, at the GV stage, hOrai1-mRFP was highly enriched at the PM, where it formed a near-perfect ring around the cell (Figure 4E), whereas at the MII stage, even though hOrai1-mRFP was still present there, its presence was weaker

(Figure 4F); this apparent reduction of hOrai1-mRFP at the PM was accompanied by increased fluorescence in the subcortical area (Figure 4F; line and bar graphs below figures), suggesting recycling of the protein, as reported in *Xenopus* oocytes (Yu *et al.*, 2009, 2010). Collectively these results suggest that the molecular components of SOCE undergo cellular redistribution during maturation.

### **hStim1 puncta formation and colocalization with hOrai1 decrease during oocyte maturation**

After depletion of  $[\text{Ca}^{2+}]_{\text{ER}}$ , Stim1 undergoes oligomerization and migration to the cell cortex, nearly reaching the PM, where these aggregates, also known as "puncta," recruit and gate Orai1 (Penna *et al.*, 2008; Park *et al.*, 2009). To assess whether the ability of Stim1 to undergo puncta formation and migration to the cortex changed during oocyte maturation, we treated hStim1-YFP-expressing GV and MII oocytes with TG and observed hStim1-YFP reorganization by confocal microscopy. To estimate the proximity of hStim1-YFP puncta to the PM, we used a diffusible dye, wheat germ agglutinin–Alexa 633, to stain the PM. After treatment with TG, hStim1 in GV oocytes readily formed distinct puncta that aligned along the PM (Figure 5, C and E), whereas this ability was severely reduced at the MII stage, along with the size of the puncta (Figure 5, D and F); the line graph to the right of Figure 5, E and F, shows the reduced intensity of the puncta in MII eggs. It is worth noting that even after TG, hStim1-YFP in MII eggs displayed a reticular organization, which was not observed in GV oocytes.

Research in somatic cells shows that Stim1 and Orai1 directly interact at the PM (Penna *et al.*, 2008; Park *et al.*, 2009). Using simultaneous expression of hStim1-YFP and hOrai1-mRFP mRNAs and confocal microscopy, we examined whether the distribution of these molecules overlapped and, if so, whether this property changed during maturation. Under resting conditions, hStim1 and hOrai1 showed their expected distributions in GV oocytes and MII eggs (Figure 5, G–I and M–O, respectively), and in GV oocytes some overlap between the molecules was noticeable (Figure 5, H and I). After addition of TG, hStim1 and hOrai1 showed extensive colocalization at the GV stage (Figure 5, K and L), although this was not evident in MII eggs (Figure 5, Q and R). Collectively the data show that the organization of hStim1 and hOrai1 follows distinct but parallel redistribution during oocyte maturation, which temporally coincides with the decline in  $\text{Ca}^{2+}$  influx during this process.

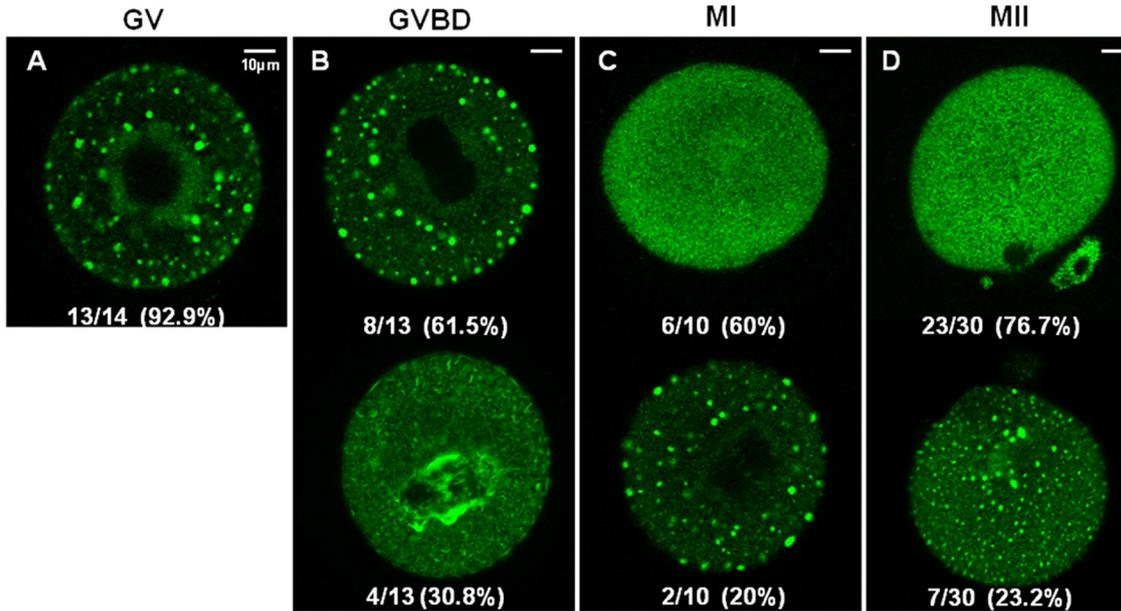
### **Coexpression of SOCE components enhances TG-induced $\text{Ca}^{2+}$ influx at all stages of oocyte maturation**

The diminished colocalization of hStim1 and hOrai1 in MII eggs after depletion of  $\text{Ca}^{2+}$  stores led us to examine whether  $\text{Ca}^{2+}$  influx was reduced at this stage. We expressed either component of SOCE alone or in combination and compared  $[\text{Ca}^{2+}]_i$  responses in GV and MII stages using the TG and  $\text{CaCl}_2$  add-back method. As shown in Figure 6A, expression of hStim1 alone enhanced influx in both stages of maturation but to a greater extent in GV than in MII, which is consistent with our previous results. Expression of hOrai1, on the other hand, failed to modify influx at either stage, whereas hStim1 and hOrai1 coexpression increased  $\text{Ca}^{2+}$  influx in both stages, although the  $[\text{Ca}^{2+}]_i$  rise was considerably smaller in MII eggs, consistent with the reduced colocalization of hStim1 and hOrai1 at this stage.

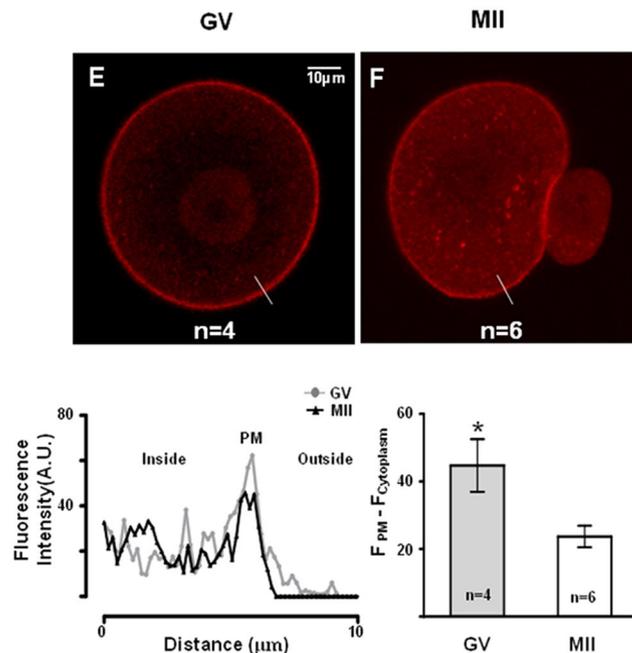
### **Expression of SOCE components alters basal $\text{Ca}^{2+}$ homeostasis during oocyte maturation**

We next examined whether expression/coexpression of the SOCE components changed basal  $[\text{Ca}^{2+}]_i$  levels under natural regular

## hStim1-YFP



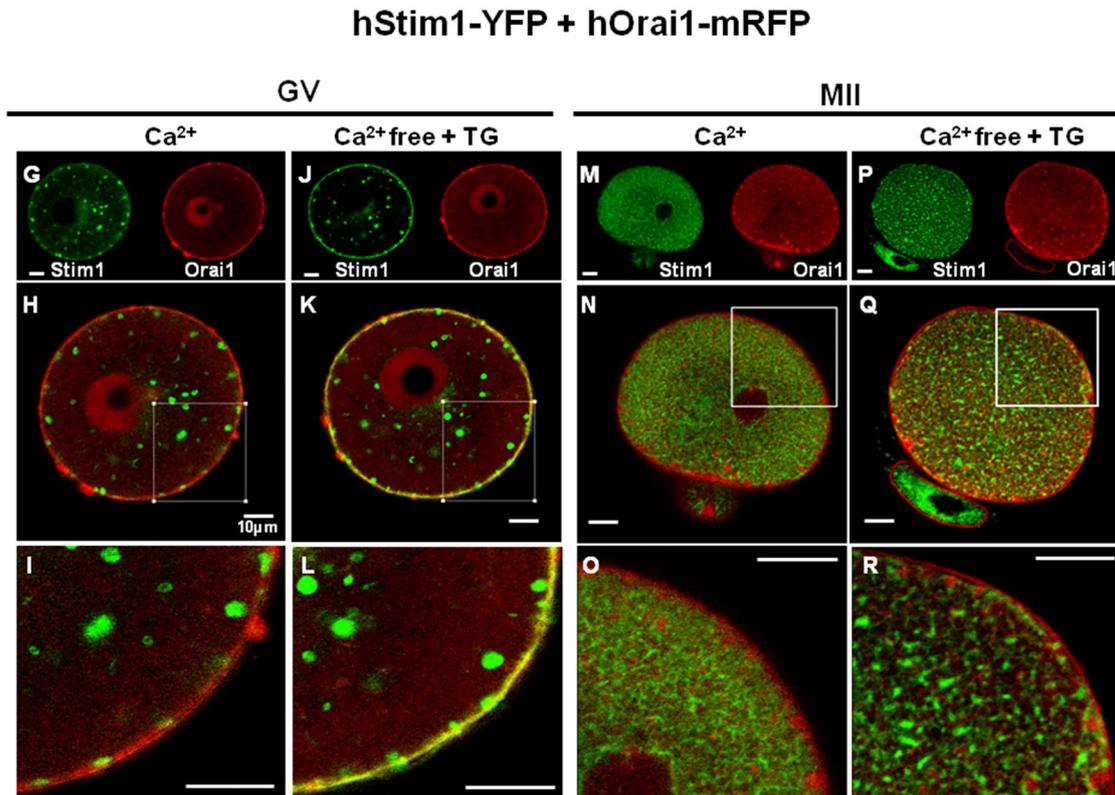
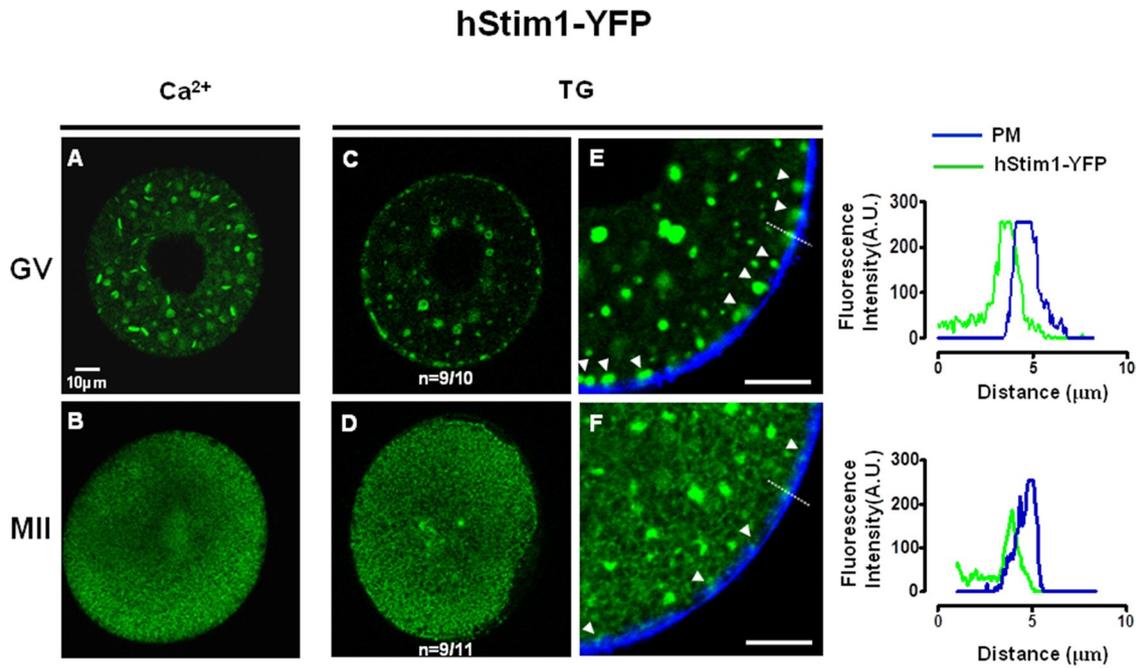
## hOrai1-mRFP



**FIGURE 4:** The distribution and organization of hStim1-YFP and hOrai1-mRFP change during oocyte maturation. (A–D) The distribution of hStim1-YFP was examined using confocal microscopy from images taken at the equatorial plane of live hStim1-YFP-expressing oocytes. The number of oocytes examined at each stage is shown at the bottom of each representative image. hStim1-YFP displayed two distinct patterns of organization in all stages of maturation except the GV stage; the most representative configurations are shown, along with the proportion of oocytes/eggs exhibiting the particular pattern. (E, F) The distribution of hOrai1 was examined as in the foregoing in GV and MII oocytes expressing hOrai1-mRFP. Intensity profiles of the line scans drawn in oocytes and eggs are shown below E, and a bar graph displaying the relative intensity of Orai1 signal between PM and cytoplasm is shown below F. Scale bar, 10  $\mu\text{m}$ .

conditions, that is, without emptying the stores and under normal external  $\text{Ca}^{2+}$  concentration,  $\sim 1.7 \text{ mM CaCl}_2$ . In control GV oocytes, basal  $[\text{Ca}^{2+}]_i$  remained steady, as the fluorescence ratio of  $\sim 0.1$  remained unchanged until the MII stage (Figure 6B, top, left).

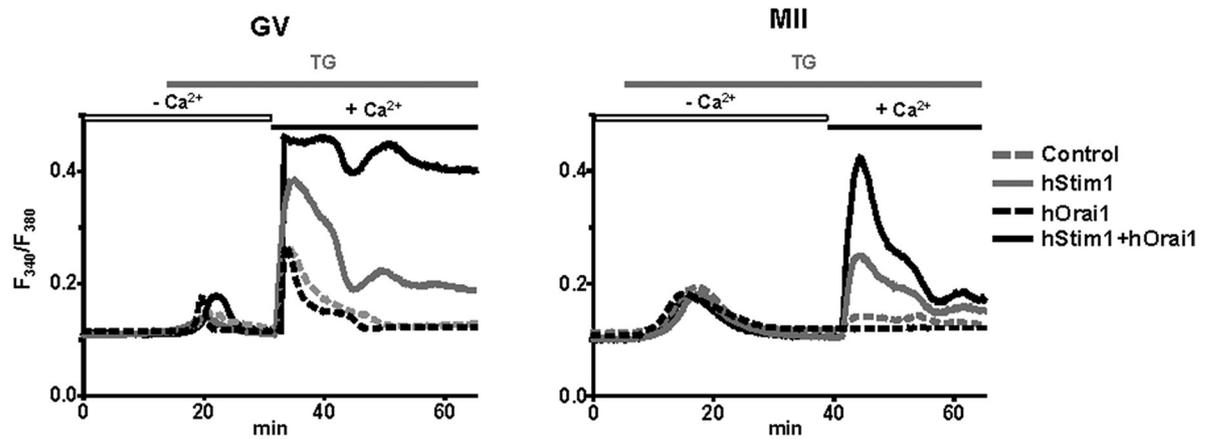
Expression of hStim1 increased baseline  $[\text{Ca}^{2+}]_i$  in GV oocytes ( $p < 0.05$ ), although by the MII stage basal  $[\text{Ca}^{2+}]_i$  had returned to levels that were indistinguishable from those of noninjected controls (Figure 6B, top, right). Coexpression of hStim1 and hOrai1



**FIGURE 5:** hStim1 puncta formation and colocalization with hOrai1 decreases during oocyte maturation. (A, B) Under control conditions, hStim1-YFP forms internal aggregates in GV oocytes, whereas in MII eggs it acquires a more homogenous, ER-like organization. After depletion of  $[Ca^{2+}]_{ER}$ , hStim1-YFP undergoes “puncta” formation in GV oocytes (C), whereas hStim1-YFP distribution is hardly changed in MII oocytes (D). Arrowheads point to hStim1-YFP puncta (E, F), and line graphs depicting the fluorescence intensity of these puncta, marked with a broken line, are shown to the right of E and F. (E, F) The proximity of the hStim1-YFP puncta/aggregates to the PM was estimated by staining the PM with wheat germ agglutinin–Alexa 664. hStim1-YFP puncta are bigger and more numerous in GV oocytes than in MII eggs. (G–R) Confocal images before and after TG treatment of GV oocytes (G–L) and MII eggs (M–R) expressing hStim1-YFP+hOrai1-mRFP. The same oocytes/eggs were imaged before and after TG. Top, separate fluorescent channels; middle, merged images; bottom, amplified regions of these are. hStim1 and hOrai1 display extensive overlap after the addition of TG in GV oocytes (K, L), but the overlap is negligible at the MII stage (Q, R). Scale bar, 10  $\mu$ m.

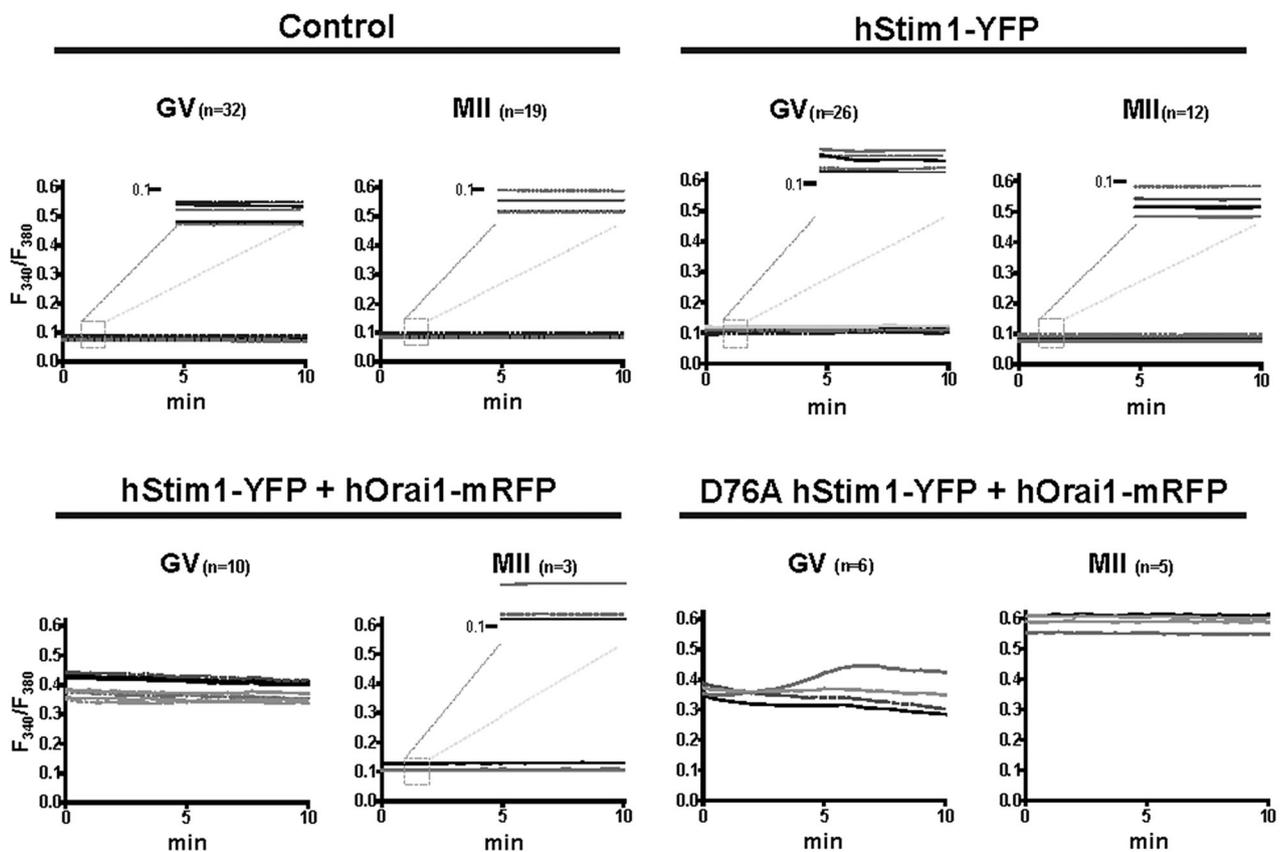
A.

### Ca<sup>2+</sup> influx



B.

### Basal [Ca<sup>2+</sup>]<sub>i</sub> levels



**FIGURE 6:** Overexpression of SOCE components alters Ca<sup>2+</sup> homeostasis. (A) Expression of hStim1-YFP+hOrai1-mRFP increased Ca<sup>2+</sup> influx in both GV and MII cells after TG and CaCl<sub>2</sub> add-back, although the increase was greater and more prolonged in GV oocytes. Expression of hStim1-YFP also increased Ca<sup>2+</sup> influx in both GV and MII stages but to a lesser extent, whereas hOrai1 had no effect. (B) Expression of hStim1 or hStim1+hOrai1 differentially increased basal [Ca<sup>2+</sup>]<sub>i</sub> during maturation. Baseline [Ca<sup>2+</sup>]<sub>i</sub> traces in control (top, left), hStim1-YFP-expressing (top, right), hStim1-YFP+hOrai1-mRFP-expressing (bottom, left), and D76A hStim1-YFP+hOrai1-mRFP-expressing (bottom, right) oocytes. Insets in each treatment depict a magnified version of the y-axis at the 0.1 mark so that minor differences in basal [Ca<sup>2+</sup>]<sub>i</sub> between GV and MII stages can be appreciated for some of the treatments.

dramatically increased basal  $[Ca^{2+}]_i$  at the GV stage ( $p < 0.05$ ), and although basal  $[Ca^{2+}]_i$  levels were reduced by the MII stage, they remained higher than for controls (Figure 6B, bottom, left). Taken together, these results demonstrate that spontaneous  $Ca^{2+}$  influx is differentially regulated during mouse oocyte maturation and is greatest at the GV stage, consistent with the presence of spontaneous oscillations at this stage (Carroll and Swann, 1992).

To ascertain whether the downturn of  $Ca^{2+}$  influx was likely due to increasing levels of  $[Ca^{2+}]_{ER}$ , we expressed an EF-hStim1 mutant, D76A-hStim1, which is insensitive to  $[Ca^{2+}]_{ER}$  levels (Liou et al., 2005). Coexpression of D76A-hStim1+hOrai1 increased basal  $[Ca^{2+}]_i$  in GV oocytes, but, unlike the case for previous treatments, basal  $[Ca^{2+}]_i$  did not decrease, but effectively increased, as oocytes progressed to the MII stage ( $p < 0.05$ ; Figure 6B, bottom, right).

### Regulation of $Ca^{2+}$ influx is required to complete oocyte maturation

We then examined whether the distinct basal  $[Ca^{2+}]_i$  profiles generated by expression of one or both of the SOCE components had differential effects on the rates of in vitro maturation. To accomplish this, we momentarily maintained control GV oocytes or oocytes injected with the selected cRNAs at the GV stage to allow for protein translation, after which in vitro maturation proceeded for 14–16 h. The majority of control oocytes, ~80%, resumed meiosis and reached the MII stage (Figure 7A), although oocytes expressing hStim1 or hStim1+hOrai1, which displayed elevated basal  $[Ca^{2+}]_i$  at the GV stage, showed reduced rates of maturation, with more oocytes remaining arrested at the GV stage ( $p < 0.05$ ). Remarkably, oocytes expressing D76A hStim1+hOrai1, which displayed persistent elevation of basal  $[Ca^{2+}]_i$ , failed to consistently resume meiosis (Figure 7A).

To examine whether the excessive  $Ca^{2+}$  influx caused by coexpression of D76A hStim1+hOrai1 was responsible for the GV arrest, we repeated the foregoing experiment but 2.5 h after release from IBMX oocytes were transferred to medium containing 0.4 mM  $CaCl_2$ . Under normal  $[Ca^{2+}]_e$ , as expected, all control oocytes reached the GVBD stage by 2.5 h, although only ~25% of oocytes expressing D76AhStim1+hOrai1 did (Figure 7B;  $p < 0.05$ ). Subsequently, all D76A-hStim1+hOrai1-expressing oocytes maintained in normal  $[Ca^{2+}]_e$ -containing media remained arrested at the GV stage, whereas cohort oocytes transferred to 0.4 mM  $CaCl_2$ -containing media progressively underwent GVBD, and by 12 h, ~50% had undergone GVBD (Figure 7C); progression of maturation in control oocytes was not affected by 0.4 mM  $CaCl_2$  media (Figure 7C). We then examined whether moving oocytes to 0.4 mM  $CaCl_2$  media altered basal  $[Ca^{2+}]_i$ . As shown in Figure 7, D and E, 2 h after changing media oocytes expressing D76A hStim1+hOrai1 displayed a significantly lower basal  $[Ca^{2+}]_i$  ( $p < 0.05$ ), whereas control oocytes were not affected by the switch. Taken together, these results suggest that regulation of  $Ca^{2+}$  influx and  $Ca^{2+}$  homeostasis play a role during normal progression of meiosis in mouse oocytes.

## DISCUSSION

In this study we examined how  $[Ca^{2+}]_e$  and  $Ca^{2+}$  influx contribute to  $[Ca^{2+}]_{ER}$  content during mouse oocyte maturation. We also investigated the presence and function of SOCE and the effect of  $Ca^{2+}$  influx misregulation on oocyte maturation. We found that whereas  $[Ca^{2+}]_{ER}$  levels increase during maturation,  $Ca^{2+}$  entry declines. We detected expression of Stim1 and Orai1 in oocytes and eggs, as well as a change in their distribution such that hStim1-YFP and hOrai1-mRFP only extensively overlapped in GV oocytes after addition of TG. Finally, expression of hStim1+hOrai1 increased basal  $[Ca^{2+}]_i$

in GV oocytes but not in MII eggs and persistently elevated basal  $[Ca^{2+}]_i$  compromised oocyte maturation. In total, our studies demonstrate that  $Ca^{2+}$  influx is closely regulated during oocyte maturation and that alteration of  $Ca^{2+}$  homeostasis undermines the completion of maturation.

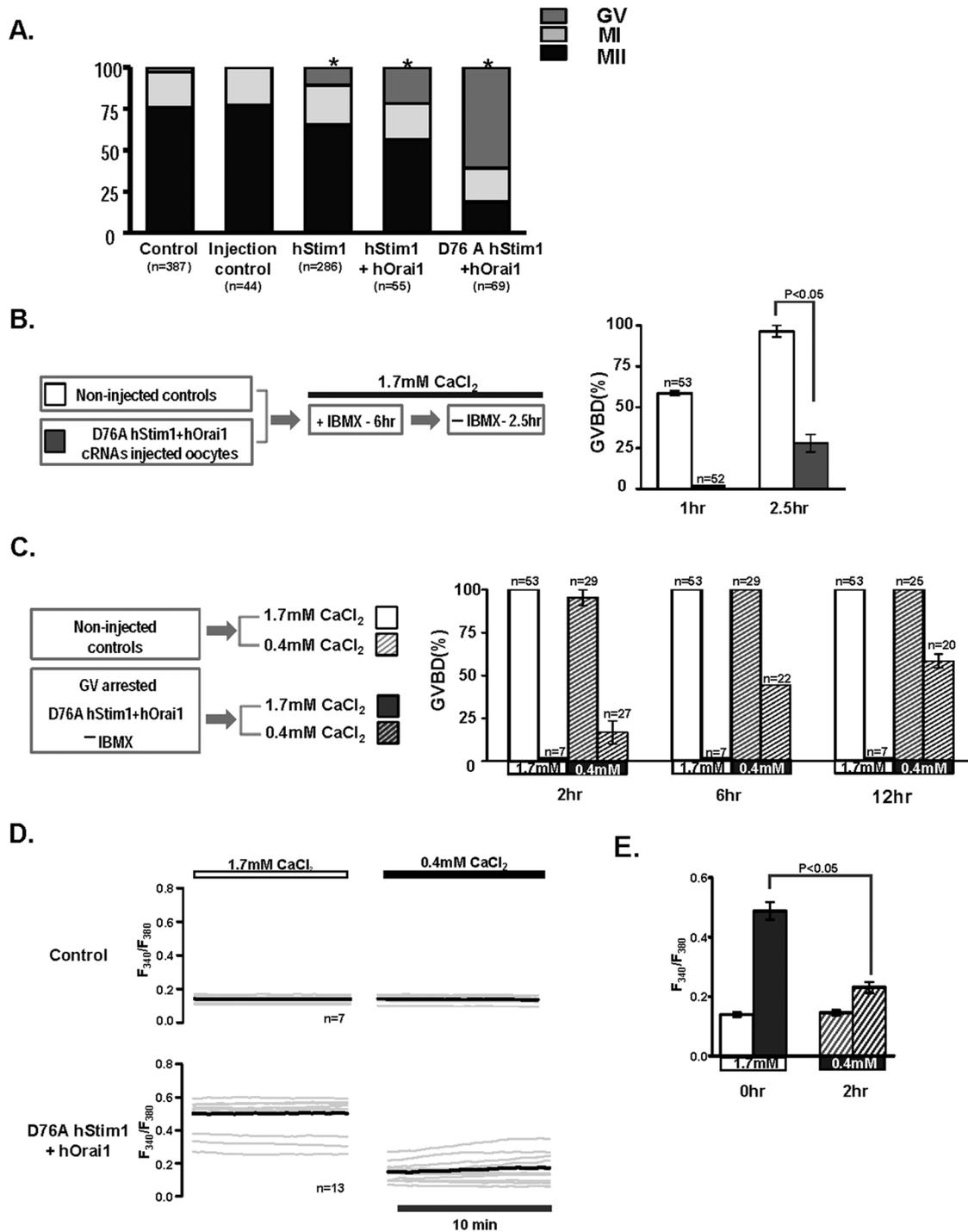
### $[Ca^{2+}]_{ER}$ , $[Ca^{2+}]_i$ influx, and SOCE during mouse oocyte maturation

Although  $[Ca^{2+}]_{ER}$  levels have been known to increase during mouse oocyte maturation, the source of  $Ca^{2+}$  and the  $Ca^{2+}$  influx mechanism(s) underlying this increase have not been established. Here we show that  $[Ca^{2+}]_e$  is required for the increase in  $[Ca^{2+}]_{ER}$  and that as  $[Ca^{2+}]_{ER}$  increases during maturation, spontaneous or TG-induced  $Ca^{2+}$  influx decreases;  $Ca^{2+}$  influx is lowest at the MII stage, which is when  $[Ca^{2+}]_{ER}$  is greatest. This relationship between  $[Ca^{2+}]_{ER}$  and  $Ca^{2+}$  influx, which is especially evident at the GV and GVBD stages, suggests participation of SOCE and is reminiscent of data for *Xenopus*, in which SOCE-mediated  $Ca^{2+}$  entry is abruptly inactivated at the GVBD stage (Machaca and Haun, 2000, 2002). Our results, unlike those for *Xenopus*, show that SOCE is only partly inactivated by the GVBD stage and can contribute to the increase in  $[Ca^{2+}]_{ER}$  during this period. Our results differ from other studies in the mouse showing progressively enhanced SOCE activity during maturation (Gomez-Fernandez et al., 2009, 2012).

Several additional findings in our study suggest a role for SOCE during mouse oocyte maturation. For example, addition of 2-APB, a broad-spectrum SOCE inhibitor (DeHaven et al., 2008; Putney, 2010), abrogated  $Ca^{2+}$  influx in GV oocytes. Moreover, both Stim1 and Orai1 were produced by oocytes throughout maturation, and expression of functional hStim1 caused enhanced  $Ca^{2+}$  influx, whereas an inactive form, hStim1- $\Delta$ CAD-YFP, failed to promote  $Ca^{2+}$  influx. In spite of such findings indicating a role of SOCE during oocyte maturation, the progressive inactivation of SOCE during maturation raises questions regarding its contribution to  $Ca^{2+}$  influx during fertilization. Recent research in mouse eggs suggests a minor role for SOCE during fertilization, for, although it was functional in MII eggs, abrogating its function by pharmacological and molecular means failed to modify sperm-initiated oscillations (Miao et al., 2012; Takahashi et al., 2013). Of importance, in porcine oocytes, inactivation of SOCE by small interfering RNA against Stim1 and Orai1 inhibited persistent fertilization-associated oscillations (Lee et al., 2012; Wang et al., 2012). Taken together, these results suggest that in mammals different  $Ca^{2+}$  influx mechanisms regulate  $[Ca^{2+}]_{ER}$  during maturation and  $Ca^{2+}$  influx during fertilization, and future studies should identify these channels and their regulation.

### Reorganization of hStim1 and hOrai1 during oocyte maturation

The inactivation of spontaneous and SOCE-mediated  $Ca^{2+}$  influx led us to examine whether changes in the distribution of Stim1 and Orai1 could be undermining  $Ca^{2+}$  influx. Given the inconsistent results obtained using immunofluorescence to localize endogenous Stim1 (data not shown), we used corresponding mRNAs also encoding fluorescent proteins. Marked changes in the organization of these proteins began around GVBD. For example, in GV oocytes internal "patches" and peri-GV accumulation highlighted the widespread distribution of hStim1, but after GVBD and for the rest of maturation, hStim1 distribution became diffuse and acquired a reticular, ER-like pattern consistent with its localization. Further, addition of TG, which in GV oocytes caused hStim1 to diffuse to the cortex and form distinct puncta, hardly changed the distribution of hStim1 in eggs. The distribution of Orai1 also changed, as in GV



**FIGURE 7:** Changes in  $\text{Ca}^{2+}$  homeostasis affect resumption of meiosis and oocyte maturation. (A) Control, sham-injected, and oocytes expressing hStim1-YFP, hStim1-YFP+hOrai1-mRFP, or D76A Stim1-YFP+hOrai1-mRFP were in vitro matured and their maturation rates assessed. Expression of any mRNAs that increased basal  $[\text{Ca}^{2+}]_i$ , even transiently caused a reduction in maturation rates, although expression D76A hStim1-YFP+hOrai1-mRFP nearly completely prevented resumption of meiosis. Asterisks above bars represent treatments that significantly reduced GVBD rates ( $p < 0.05$ ). (B) The effect of coexpression of D76A hStim1-YFP + hOrai1-mRFP on meiotic resumption was investigated as depicted in the flow chart (left), and data are summarized in the bar graph (right). Expression of D76A hStim1-YFP+hOrai1-mRFP in GV oocytes delayed and mostly prevented GVBD under normal  $[\text{Ca}^{2+}]_e$  ( $p < 0.05$ ). (C) As before, but selected GV-arrested oocytes were transferred to low- $[\text{Ca}^{2+}]_e$  medium (0.4 mM), which partly rescued the arrest caused by expression of D76A hStim1-YFP+hOrai1-mRFP. (D)  $\text{Ca}^{2+}$  traces depicting the effect of lowering  $[\text{Ca}^{2+}]_e$  on basal  $[\text{Ca}^{2+}]_i$  in control and GV-arrested D76A hStim1+hOrai1-expressing oocytes monitored before and 2 h after lowering  $[\text{Ca}^{2+}]_e$  from 1.7 to 0.4 mM; the bold trace represents the mean of the responses. (E) A bar graph was used to summarize in the same group of oocytes the mean  $\pm$  SEM change in basal  $[\text{Ca}^{2+}]_i$  caused by lowering external  $[\text{Ca}^{2+}]_e$ ;  $p < 0.05$ .

oocytes it was primarily present on the PM, but by MII its PM presence was reduced and its signal in the ooplasm increased. In agreement with these changes, after addition of TG, hStim1 and hOrai1 only colocalized on the PM of GV oocytes. Our results are in agreement with those in *Xenopus* oocytes, where inactivation of SOCE during maturation was associated with inhibition of Stim1 oligomerization/puncta formation and Orai1 internalization (Yu *et al.*, 2009, 2010). Nevertheless, our results show that hStim1 retained certain clustering capacity and Orai1 internalization was incomplete in MII eggs, as even at this stage expression of hStim1+hOrai1 enhanced SOCE activity. Our results also concur with data in somatic cells, where SOCE is down-regulated at mitosis (Russa *et al.*, 2008; Smyth *et al.*, 2009) and this change was associated with reduced ability of Stim1 to form puncta (Smyth *et al.*, 2009). Data from studies in mammalian oocytes also support our findings. For example, after depletion of  $[Ca^{2+}]_{ER}$  Stim1 displayed some degree of oligomerization in porcine MII eggs (Koh *et al.*, 2009), and in mouse eggs Stim1 showed conspicuous clusters/puncta organization, although without emptying of the stores, and Orai1 distribution was mostly concentrated at the PM (Gomez-Fernandez *et al.*, 2012).

We did not investigate the mechanisms that regulate Stim1 reorganization in oocytes. Nevertheless, Stim1 was originally described as a phosphoprotein (Manji *et al.*, 2000), and phosphorylation of its S/T-P consensus sites by M-phase kinases was demonstrated in somatic cells (Manji *et al.*, 2000; Smyth *et al.*, 2009; Pozo-Guisado *et al.*, 2010) and *Xenopus* oocytes (Yu *et al.*, 2009). We found that exogenous hStim1 was phosphorylated in MII eggs, as it experienced a mobility shift that was reduced by phosphatase treatment. We did not observe similar changes in the endogenous protein, although the comparable temporal inactivation of TG-induced  $Ca^{2+}$  influx between uninjected and hStim1 mRNA-injected oocytes suggest common regulatory mechanisms.

### **Ca<sup>2+</sup> influx and progression of oocyte maturation**

To gain further insight into the mechanisms underlying the brief presence of  $[Ca^{2+}]_i$  oscillations in GV stage mouse oocytes (Carroll and Swann, 1992; Carroll *et al.*, 1994), we expressed, singly or together, hStim1 and hOrai1 mRNAs and monitored their effects on basal  $[Ca^{2+}]_i$  in oocytes and eggs. We found that their combined expression markedly and persistently increased basal  $[Ca^{2+}]_i$  in GV oocytes, although by the MII stage, basal  $[Ca^{2+}]_i$  levels had returned to near-normal levels. These results suggest close regulation of  $Ca^{2+}$  homeostasis/influx during the early stages of maturation.

To better understand the role of basal  $[Ca^{2+}]_i$  and  $Ca^{2+}$  influx on oocyte maturation, we coexpressed D76A hStim1+hOrai1 mRNAs, which increased basal  $[Ca^{2+}]_i$  throughout maturation. Under these conditions, progression of maturation was greatly reduced, with most oocytes remaining at the GV stage. These effects were due to the inability to inactivate  $Ca^{2+}$  influx and decrease basal  $[Ca^{2+}]_i$ , as lowering external  $[Ca^{2+}]_e$  reduced  $[Ca^{2+}]_i$  and rescued the ability of these oocytes to undergo GVBD. Similar detrimental effects of elevated basal  $[Ca^{2+}]_i$  were observed in *Xenopus* oocytes, where enhanced  $Ca^{2+}$  influx promoted throughout maturation hindered the progression of meiosis (Sun and Machaca, 2004). Therefore it appears that regulation of  $Ca^{2+}$  influx is required for normal progression of oocyte maturation in the mouse, although we did not explore the functional mechanism(s) positively affected by the progressive inactivation of  $Ca^{2+}$  entry.

We propose a model in which mouse GV oocytes exhibit a low but persistent  $Ca^{2+}$  influx that contributes to the GV's overall cellular metabolism. On resumption of meiosis,  $Ca^{2+}$  influx is progressively inactivated to allow for normal progression of meiosis, spindle

organization, and MII arrest. Future studies in mammalian oocytes should elucidate the mechanism(s) responsible for the inactivation of  $Ca^{2+}$  influx and the identity of the channel(s) that mediate  $Ca^{2+}$  entry during maturation and fertilization.

## **MATERIALS AND METHODS**

### **Collection and preparation of mouse oocytes**

Fully grown GV-stage oocytes were collected from the ovaries of 6- to 10-wk-old CD-1 female mice 44–46 h after injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich, St. Louis, MO; all chemicals are from Sigma-Aldrich unless otherwise indicated), as we previously described (Kurokawa *et al.*, 2007). Cumulus intact GVs were recovered into a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Tyrode's lactate solution (TL-HEPES) containing 5% fetal calf serum (Invitrogen, Carlsbad, CA) and supplemented with 100  $\mu$ M IBMX to block spontaneous progression of meiosis. When necessary, cumulus cells were removed by repetitive pipetting using a fine capillary glass. Oocytes were matured in vitro for 12–14 h in IBMX-free Chatot, Ziomek, and Bavister medium (CZB; Chatot *et al.*, 1989) supplemented with 3 mg/ml bovine serum albumin (BSA) or 0.02% polyvinyl alcohol (average molecular weight 30,000–70,000) under paraffin oil at 36.5°C in a humidified atmosphere containing 6% CO<sub>2</sub>. In vivo-matured MII oocytes were collected from the oviducts 12–14 h after administration of 5 IU of human chorionic gonadotropin, which was injected 46–48 h after PMSG. All procedures were performed according to research animal protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee.

### **Generation of constructs and mRNA preparation**

hStim1-YFP and hOrai1 were generously provided by T. Meyer (Stanford University, Stanford, CA) and M. Trebak (Albany Medical College, Albany, NY), respectively. hStim1-YFP was subcloned into a pcDNA6/Myc-His B vector (Invitrogen) between the restrictions sites AgeI and XbaI. The hOrai1 insert was amplified by PCR and ligated to the N-terminus of the mRFP-bearing pcDNA6/Myc-His B vector (D. Alfandari, University of Massachusetts, Amherst, MA) between EcoRI and XhoI restriction sites. Constitutively active hStim1, D76A hStim1-YFP, and hStim1-482 stop were generated either by substituting D76 to A or introducing the stop codon after amino acid 481 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), as previously reported (Liou *et al.*, 2005; Smyth *et al.*, 2009). Before performing in vitro transcription reactions, we verified the sequences of all new constructs and presence of targeted mutations by DNA sequencing. Constructs were linearized outside of the coding region with PmeI and in vitro transcribed using T7 mMES-SAGE mMACHINE Kit (Ambion, Austin, TX). A poly(A) tail was added to the mRNAs using a Tailing Kit (Ambion). All mRNAs were prepared to final concentrations of 1.5  $\mu$ g/ $\mu$ l, aliquoted, and frozen at –80°C until use.

### **Microinjection of mRNAs**

Microinjections were performed as described previously by our laboratory (Kurokawa *et al.*, 2007). Before injection, cRNAs were heat denatured and centrifuged, and the top 1.2  $\mu$ l was used to prepare microdrops from which glass micropipettes were loaded by aspiration. cRNAs were delivered into oocytes using pneumatic pressure (PLI-100 picoinjector; Harvard Apparatus, Holliston, MA). When cRNAs were injected simultaneously, as in the case of hOrai1-mRFP+hStim1-YFP or hOrai1-mRFP+D76A hStim1-YFP, cRNAs were mixed immediately before the injection procedure in 1–3 M ratios, respectively, to allow similar protein expression, which

was estimated by comparing fluorescence intensities. When injections were performed in GV oocytes and to allow for maximal translation, oocytes were kept in CZB plus IBMX for 6 h, after which IBMX was removed to allow the commencement of maturation. GVBD- and MI-stage oocytes were matured for 4 and 8 h, respectively, whereas MII oocytes were matured for 12–14 h in CZB.

### [Ca<sup>2+</sup>]<sub>i</sub> measurements and Ca<sup>2+</sup> reagents

[Ca<sup>2+</sup>]<sub>i</sub> monitoring was performed as previously reported by our laboratory (Kurokawa *et al.*, 2007). In brief, Fura-2 acetoxymethyl ester (Fura-2AM) was loaded by incubating oocytes in a HEPES-buffered CZB solution (HCZB) containing 1.25 μM Fura-2AM for 20 min at room temperature. Oocytes were then immobilized on glass-bottom dishes (MatTek Corp., Ashland, MA) and placed on the stage of an inverted microscope (Nikon, Melville, NY). Fura-2 fluorescence was excited with 340- and 380-nm wavelengths every 20 s, and emitted light was collected at wavelengths >510 nm by a cooled Photometrics SenSys charge-coupled device camera (Roper Scientific, Tucson, AZ). Acquisition of fluorescence ratios and rotation of the filter wheel were controlled by Simple PCI software (C-Imaging System Software, Cranberry Township, PA).

To examine the role of Ca<sup>2+</sup> influx on refilling of [Ca<sup>2+</sup>]<sub>ER</sub>, we placed IBMX-treated GV oocytes in CZB medium either with or without 1.7 mM CaCl<sub>2</sub> and allowed them to mature for 4 h, after which we placed them in nominal Ca<sup>2+</sup>-free HCZB and after a 5-min interval assessed interval [Ca<sup>2+</sup>]<sub>ER</sub> levels by adding TG (Calbiochem, San Diego, CA), an inhibitor of the SERCA pump, which induced Ca<sup>2+</sup> leak by an unknown mechanism. TG-induced Ca<sup>2+</sup> rises were regarded as [Ca<sup>2+</sup>]<sub>ER</sub> content that could be estimated from the area under the curve of the [Ca<sup>2+</sup>]<sub>i</sub> rise using Prism software (GraphPad Software, La Jolla, CA). To estimate SOCE and assess the effect of [Ca<sup>2+</sup>]<sub>ER</sub> on hStim1-YFP distribution, we followed oocytes by the method in Bird *et al.* (2008). Before adding 10 μM TG, we placed oocytes in Ca<sup>2+</sup>-free HCZB supplemented with 1 mM ethylene glycol tetraacetic acid (EGTA). When [Ca<sup>2+</sup>]<sub>i</sub> returned to near baseline values, ~35 min after TG addition, 5 mM CaCl<sub>2</sub> was added to the medium, and the amplitude of the [Ca<sup>2+</sup>]<sub>i</sub> rise caused by the addition was used to estimate SOCE.

### Western blotting procedures

To detect endogenous/exogenous Stim1 and Orai1, we prepared protein lysates from 200 or 20–45, respectively, GV or MII oocytes. Oocytes were washed in Dulbecco's phosphate-buffered saline (DPBS), lysed in 2× sample buffer, and stored at –20°C until use. Heat-denatured proteins (95°C for 3 min) were separated by 7.5 or 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 6% skim milk dissolved in PBS plus 0.1% Tween-20 for 2 h at 4°C. Two different antibodies were used against Stim1, one raised to recognize the N-terminus (1:100; BD Biosciences, San Jose, CA) and the other to identify the C-terminus end of the molecule (1:500; ProSci, Poway, CA), for which a blocking antigenic peptide (AP) was available. Orai1 was detected using an anti-Orai1 antibody (1:300; ProSci) raised against the C-terminus of the molecule, and an AP was also available for this antibody. For these experiments, equal volumes of AP (ProSci) and of the specific antibody were incubated for 2 h at 4°C, after which this mixture was used to complete the Western blotting procedure. In all samples, an anti-actin antibody was used to detect actin reactivity, which was used as a loading control (1:500; Millipore). Blots were incubated overnight at 4°C with primary antibodies, and goat anti-mouse or anti-rabbit antibodies conjugated with horseradish peroxidase were

used as secondary antibodies (1:2000; Bio-Rad, Hercules, CA) and incubated for 1 h at room temperature. The membranes were then exposed to chemiluminescence reagents (NEN Life Science Products, Boston, MA) and the signal assessed using a Kodak 440 Image Station (Rochester, NY). The same anti-Stim1 antibodies were used to detect exogenously expressed hStim1-YFP (*n* = 45), which were also detected using an anti-GFP antibody (1:1000; MBL, Woburn, MA).

Phosphatase treatment was carried out on wild-type hStim1-YFP-expressing GV and MII oocytes/eggs. Samples were washed in DPBS and placed in phosphatase buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN); control samples were also supplemented with 50 mM β-glycerophosphate to inhibit endogenous phosphatases. All samples were lysed by repeated cycles of freezing and thawing using liquid nitrogen, and 0.5 U of calf intestine phosphatase (New England Biolabs, Ipswich, MA) was added to the indicated groups; all samples were incubated at 37°C for 30 min. The reaction was stopped by addition of 2× sample buffer, after which Western blotting was performed as described.

### Plasma membrane staining

To estimate the proximity of hStim1-YFP puncta to the PM during the different stages of maturation, we stained the PM of oocytes/eggs using 10 μg/ml wheat germ agglutinin conjugated with Alexa Fluor 633 (Invitrogen) according to manufacturer's instructions. Before staining, the zona pellucida was removed using Tyrode's acidic solution, pH 2.5, to facilitate the diffusion of the stain. ImageJ (National Institutes of Health, Bethesda, MD) software was used to quantify the distances of the hStim1 puncta to the PM in micrometers and the diameters of hStim1 aggregates and to draw the line scan to compare hOrai1 PM distribution.

### Live imaging of oocytes using confocal microscope

Oocytes/eggs expressing proteins tagged with fluorescent proteins were collected at variable times of maturation and attached to glass-bottom dishes while incubated in BSA-free HCZB medium. Fluorescence was examined using an LSM 510 META confocal microscope (Carl Zeiss Microimaging, Jena, Germany) outfitted with a 63×/1.4 numerical aperture oil immersion lens. Images were taken at the equatorial and cortical regions of oocytes/eggs.

### Statistical analysis

Statistical analysis was performed using Prism software (GraphPad, La Jolla, CA). All data are presented as mean ± SEM. Mean data were compared using unpaired *t* test or analysis of variance, as appropriate. Categorical values such as those generated by maturation rates were analyzed using the chi-square test. *p* < 0.05 was considered significant.

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