**Obox4-silencing-activated STAT3 and MPF/MAPK signaling accelerate nuclear membrane breakdown in mouse oocytes**

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**Abstract**

Mouse oocytes begin to mature in vitro once liberated from ovarian follicles. Previously, we showed that oocyte-specific homeobox 4 (Obox4) is critical for maintaining the intact nuclear membrane of the germinal vesicle (GV) in oocytes and for completing meiosis at the metaphase I–II (MI–MII) transition. This study further examines the molecular mechanisms of OBOX4 in regulating GV nuclear membrane breakdown. Maturation-promoting factor (MPF) and MAPK are normally inactive in GV stage oocytes but were activated prematurely in arrested GV stage oocytes by 3-isobutyl-1-metyl-xanthine (IBMX) in vitro after Obox4 RNA interference (RNAi). Furthermore, signal transducer and activator of transcription 3 (STAT3) was significantly activated by Obox4 RNAi. We confirmed that this Obox4 RNAi-induced premature STAT3 and MPF/MAPK activation at the GV stage provoked subsequent GV breakdown (GVBD) despite the opposing force of high cAMP in the IBMX-supplemented medium to maintain intact GV. When cumulus–oocyte complexes were exposed to interferon α (IFNA), a STAT3 activator, oocytes matured and cumulus cells expanded to resume nuclear maturation in IBMX-supplemented medium, suggesting that STAT3 activation is sufficient for stimulating the continuation of meiosis. Using Stattic, a specific STAT3 inhibitor, we confirmed that GVBD involves STAT3 activation in Obox4-silenced oocytes. Based on these findings, we concluded that i) Obox4 is an important upstream regulator of MPF/MAPK and STAT3 signaling, and ii) Obox4 is a key regulator of the GV arrest mechanism in oocytes.

Reproduction (2016) 151 369–378

**Introduction**

In vivo, immature oocytes have large nuclei (germinal vesicles (GVs)) and are arrested at prophase I until a surge of luteinizing hormone triggers meiotic resumption (Mehlmann 2005). However, fully grown oocytes that are liberated from follicles resume meiosis spontaneously in vitro when placed in a suitable culture medium (Sato & Koide 1984). However, this spontaneous maturation is suppressed when cAMP analogs or phosphodiesterase (PDE) inhibitors, such as 3-isobutyl-1-metyl-xanthine (IBMX), are added, resulting in high levels of cAMP in the culture medium (Cho et al. 1974, Conti 2002). Knockout of the PDE3A gene similarly blocks maturation, preventing meiotic resumption (Masciarelli et al. 2004).

We previously reported that GV stage arrest is not sustained in oocyte-specific homeobox 4 (Obox4) RNAi-treated oocytes, and nuclear membrane breakdown (termed GV breakdown (GVBD) in oocytes) occurs in Obox4-silenced oocytes, even in IBMX-supplemented medium (Lee et al. 2010). Although the molecular mechanisms involved were unclear, Obox4 knockdown stimulated oocytes to resume meiosis in IBMX-supplemented medium.

High cAMP levels maintain downstream protein kinase A (PKA) in an active/dissociated state, and activated PKA is critical for sustained meiotic arrest (Bornslaeger et al. 1986). Moreover, high PKA activity decreases MPF activity and vice versa. This effect is linked to a signaling network involving Cdc25 phosphorylation and cyclin B degradation (Grieco et al. 1994). MPF activity is regulated primarily by the phosphorylation of two highly conserved residues (Thr14 and Tyr15) of Cdc2, and both residues undergo phosphorylation by Wee1 kinase (Parker & Piwnica-Worms 1992, Fattaey & Booher 1997, Barbacid et al. 2005). Substantial evidence strongly suggests that PKA regulates Cdc25 and Wee1 through the phosphorylation of Serine 287 and Serine 549 respectively, whereas MPF...
activity is self-regulated after activating Cdc25 and inactivating Wee1 (Bornslaeger et al. 1986, Duckworth et al. 2002, Han et al. 2005). Although oocyte meiotic arrest unquestionably relies on a cAMP-dependent cascade, the broader effects of this cascade are unknown. Neither the maintenance of meiotic arrest nor the resumption of meiosis has been adequately characterized to date.

Various reports have indicated that many signal transduction pathways are involved in GVBD. Signal transduction proteins, such as MAPK (ERK1/2, JNK and p38) and signal transducer and activator of transcription 3 (STAT3), are known to be phosphorylated during oocyte maturation (Yoon et al. 2009). The MAPK cascade is among the principal regulatory systems driving oocyte meiotic progression and acts in parallel and jointly with MPF in this regard (Villa-Diaz & Miyano 2004). Likewise, in mature oocytes, STAT3 is differentially distributed in the cytoplasm and is present in pre-implantation embryos (Antczak & Van Blerkom 1997). Changes in STAT3 phosphorylation in response to the maturation status of oocytes were documented by Yoon et al. (2009). In a rabbit model, the JAK2/STAT3 and MEK1/2 pathways mediate nuclear maturation by leptin but do not mediate the cytoplasmic maturation or steroidogenesis of the cumulus–oocyte complex (COCs) (Arias-Alvarez et al. 2010). However, STAT3 knockout is lethal for embryonic development in animals (Takeda et al. 1997). In this study, we examined the relationship between OBOX4 and GVBD induction by investigating the involvement of the STAT3 and MPF/MAPK signaling pathways after Obox4 RNAi in mouse oocytes.

Materials and methods

Animals

Imprinting control region (ICR) mice were obtained from Koatech (Pyeoungtack, Korea) and served as oocyte reservoirs. The mice were maintained at the animal facility of the CHA Stem Cell Institute of CHA University. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of CHA University and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Oocyte collection

To collect GV stage oocytes from preovulatory follicles, 3-week-old female ICR mice were injected with 5 IU of pregnant mare serum gonadotropin (Sigma) and killed 46 h post-eCG injection. The removed ovaries were then submerged in M2 medium (Sigma) containing 0.2 mM IBMX (Sigma) to inhibit the GVBD of oocytes released from the follicles. We used IBMX, a phosphodiesterase inhibitor, to maintain a high concentration of cAMP levels in the cells because high cAMP concentrations importantly regulate the maintenance of intact GV in vitro (Cho et al. 1974). The COCs were also recovered in the IBMX-supplemented medium, and the cumulus cells (CCs) were removed from the COCs by aspiration with a fine-bore pipette.

Preparation and microinjection of dsRNA

To generate a template for in vitro transcription, 2 μg of total RNA from adult ovaries were reverse-transcribed using M-MLV reverse transcriptase (RT) (Promega) and amplified using Obox4-specific primers, as described previously (Lee et al. 2010). The Obox4 cDNA fragment was cloned into the pGEM T-easy vector (Promega), and RNA was synthesized using T7 polymerase and a MEGAscript kit (Ambion, Austin, TX, USA). The primers used to amplify Obox4 dsRNA are shown in Table 1. In-house single-stranded RNAs were used to prepare annealed, purified Obox4 dsRNA, and the specific suppression of Obox mRNA expression was confirmed by ensuring the downregulation of target gene expression, but not of other genes that are functionally or structurally unrelated to the target Obox (Supplement 1A and B, see section on supplementary data given at the end of this article).

Denuded oocytes with intact GVs were microinjected with Obox4 dsRNA and placed in M2 medium containing 0.2 mM IBMX (Sigma). Approximately 10 pl of dsRNA (2.5 μg/μl) was microinjected into the cytoplasm of the oocytes using a constant-flow system (Femtojet; Eppendorf, Hamburg, Germany). These microinjected oocytes were then cultured in M16 medium (Sigma) containing 3 mg/ml BSA (Sigma) in the presence or absence of 0.2 mM IBMX (Sigma) with 5% CO2 at 37 °C for 4 or 8 h. In our previous studies, we found that 2–4 h of incubation is sufficient for Obox4 transcript knockdown (Lee et al. 2010). To confirm the specificity of the Obox4–STAT3 interaction, Obox4-silenced GV oocytes were cultured in M16 medium containing 3 mg/ml BSA and 0.2 mM IBMX with 5% CO2 at 37 °C for 8 h with 1 μM Stat6 (a non-peptidic small molecule that selectively inhibits STAT3 function) (Schust et al. 2006). Oocytes without GV or polar bodies were marked as GVBD.

mRNA isolation

mRNA was isolated from the same numbers of oocytes using a Dynabeads mRNA DIRECT kit (Invitrogen). The oocytes were suspended in 300 μl lysis-binding buffer for 5 min at room temperature (RT). After vortexing, 20 μl prewashed Dynabeads oligo dT25 was mixed with the lysate, and the mRNA was annealed by rotating for 5 min at RT. The beads were separated using a Dynal MPC-5 magnetic particle concentrator, and poly (A)+ RNAs were eluted by incubating the beads in 10 μl of 10 mM Tris–HCl, pHe 7.5, at 65 °C for 2 min. The eluted mRNA was used for reverse transcription.

RT-PCR analysis

Total RNA and 0.5 pg oligo (dT) primer were mixed and incubated at 70 °C for 10 min; cDNA was then synthesized according to the manufacturer’s protocol. The reverse transcription reaction was (in brief) carried out in a final volume of
20 μl M-MVL RT 5X buffer containing 0.5 mM dNTPs and 200 U of M-MLV RT (Promega). The reaction mixture was incubated at 42 °C for 90 min and then at 94 °C for 2 min. cDNA equivalent to that obtained from one oocyte served as a template for PCR analysis. The PCR primers used are listed in Table 1. Following PCR, 20-μl aliquots of each sample were resolved using electrophoresis on a 1.5% agarose gel, and the results were recorded using an Image Analyzer.

### Isolation and culture of COCs in vitro

COCs that were recovered from preovulatory follicles by needle puncture of the ovary were collected in M2 medium (Sigma) containing 0.2 mM IBMX (Sigma) to maintain the oocytes at the GV stage. Thereafter, the COCs were cultured in IBMX-supplemented M16 medium (Sigma) containing 3 mg/ml BSA (Sigma) at 37 °C in a 5% CO2 incubator. After 22 h of culture, the oocytes were denuded by repeatedly drawing the COCs into and out of a small bore pipette and were then examined under a microscopic scope to determine the progression of meiotic maturation. To determine in vitro expansion, the COCs were allocated to separate wells of a Nunc 4-well plate in 100 μl M16 medium under a mineral oil cover in the absence of IFNA (5000 unit/ml, Sigma). After 22 h of culture, the oocytes were evaluated using the iCycler iQ real-time detection system (Bio-Rad). iQ SYBR Green Supermix PCR reagents (Bio-Rad) were used to monitor amplification, and the results were evaluated using the iCycler iQ real-time detection system software. Gene amplification was quantitated by determining the cycle threshold (C_T) based on the level of fluorescence detected within the geometric region of the semi-log amplification plot. The expression of each mRNA species was normalized to that of H1foo mRNA. The relative quantitation of target gene expression was evaluated using the comparative C_T method, and experiments were repeated at least three times. The real-time PCR conditions and primer sequences used are listed in Table 1.

### Immunofluorescence staining

Oocytes were fixed in paraformaldehyde (PFA) solution (4% PFA, 0.2% Triton X-100) for 40 min at RT and then washed in polyvinyl alcohol (PVA)–PBS for 10 min three times for overnight storage in 1% BSA–PBS–PVA (1% BSA in PBS–PVA) at 4 °C. The oocytes were blocked with 3% BSA–PBS–PVA for 1 h and then incubated in 1% BSA–PBS–PVA containing mouse monoclonal tubulin antibody (1:100 dilution; sc-8035; Santa Cruz Biotechnology) at 4 °C overnight. After washing three times in PVA–PBS, the oocytes were incubated for 1 h at RT with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (1:100 dilution; Sigma) in 1% BSA–PBS–PVA. After a further three washes, the oocytes were incubated in PI solution (propidium iodide, 1 mg/ml; Sigma) for 20 min and then mounted on glass slides using clean coverslips.

### Western blot analysis

For protein analysis, the oocytes were washed three times with PBS. A total of 100 oocytes per lane were subjected to Western blot analysis. Oocyte lysates were produced by adding 10 μl of protein extraction solution (PRO-PREP, iNtRON Biotechnology, Gyeonggi-do, Korea) to the sampled oocytes. Proteins were separated using 12% SDS-PAGE and then transferred to a PVDF membrane (Amersham Bioscience, Piscataway, NJ, USA). The membranes were first incubated for 1 h in TBS-T (TBS-T; 0.1% Tween-20) containing 5% nonfat milk. The membranes were then incubated in 1% BSA–PBS–PVA containing mouse monoclonal tubulin antibody (1:100 dilution; sc-8035; Santa Cruz Biotechnology) at 4 °C overnight. After washing three times in PVA–PBS, the membranes were incubated for 1 h at RT with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (1:100 dilution; Sigma) in 1% BSA–PBS–PVA.

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### Table 1 Details of primers used for RT-PCR or qRT-PCR.

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<th>Genes</th>
<th>Accession no.</th>
<th>Primer sequence</th>
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<th>Size (bp)</th>
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<td>Obox4-1</td>
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<td>F-CCCTCATGATCAACCCCTTGG R-AGTTTTGGGTCATACTTGGAG</td>
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<tr>
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<tr>
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<td>NM_009398</td>
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<tr>
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<td>H1foo</td>
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<td>F-GCGAAACCGAAAGAGGTCAGAA R-TGGAGGAGGTCTTGGGAAGTAA</td>
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F and R in the primer codes indicate forward and reverse. Obox4-1 primers were used for RT-PCR and synthesis of dsRNA. Obox4-2 primers were used to confirm the knockdown of Obox4 gene expression analysis, the COCs were cultured in 100 μl M16 containing IBMX for 16, 20 and 24 h, and the isolated in vitro microscopic examination after 22 h of culture. For presence of IFNA (Sigma). Expansion was assessed by M16 medium under a mineral oil cover in the absence or presence of IFNA (Sigma).
dry milk and then incubated overnight with primary antibody (diluted 1:1000 in TBS-T containing 5% nonfat dry milk). Finally, the membranes were incubated with HRP-conjugated anti-goat secondary antibody for 1 h. The results were assessed using an ECL detection system (Santa Cruz Biotechnology). The antibodies utilized were as follows: anti-Obox4 (LabFrontier, Gyeonggi-do, Korea), anti-STAT3 (#9132, Cell Signaling Technology), anti-phospho-Tyr705-STAT3 (#9131, Cell Signaling Technology) and anti-α-Tubulin (sc-8035; Santa Cruz Biotechnology).

Dual kinase activity assay

 Mature oocytes (MI and MII) were collected at 16 or 24 h after Obox4 RNAi for dual kinase assay and were cultured in either IBMX-free or IBMX-supplemented medium, as previously described (Lee et al., 2010). For the present study, we performed a dual kinase activity assay with using immature GV stage oocytes that were collected at appropriate time points after Obox4 RNAi, followed by in vitro maturation in IBMX-supplemented medium according to the experimental design. All oocytes were then washed in 0.1% PBS–PVA, and each oocyte was placed in an Eppendorf tube with 1 μl of 0.1% PBS–PVA and 4 μl of ice-cold extraction buffer (80 mM β-glycerophosphate, 25 mM HEPES (pH 7.2), 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 1 mM APMSF, 0.1 mM Na3VO4, 1 mg/ml leupeptin and 1 mg/ml aprotinin). Each single-oocyte sample was assayed for both MPF and MAPK. Thawed oocytes were centrifuged at 13,000 g for 3 min; kinase buffer and substrate solution were then added (5 μl each), and the samples were then incubated for 20 min at 37 °C. The kinase buffer comprised 75 mM HEPES (pH 7.2), 75 mM β-glycerophosphate, 75 mM MgCl₂, 6 mM DTT, 10 mM EGTA, 60 mM ATP, 15 mM cAMP-dependent protein kinase inhibitor peptide and 0.3 μCi/μl [γ-32P]-ATP (250 μCi/25 μl; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The substrate solution used in the MPF and MAPK dual kinase assay contained 4.5 μl histone H1 (5 mg/ml, calf thymus) and 0.5 μl myelin basic protein (MBP) (5 mg/ml, bovine brain). The reaction was terminated by adding 5 μl of 4× SDS sample buffer and then boiling the sample for 5 min. Samples were resolved using 15% PAGE; the gels were then dried and autoradiographed.

Statistical analysis

Each experiment was performed independently on at least three occasions. The data were analyzed using one-way ANOVA, and the results are presented as the means ± S.E.M. A value of P<0.05 was considered statistically significant.

Results

Obox4 RNAi induced GVBD in IBMX-supplemented medium

After Obox4 knockdown in oocytes, the nuclear maturation of Obox4-silenced oocytes (GVBD) was scored during in vitro maturation for 24 h in IBMX-supplemented medium (Fig. 1A). In the presence of IBMX, the nuclear membrane of control (100%) and buffer-injected (96.6%) oocytes was maintained intact for up to 24 h of incubation; 39.6% of the Obox4 RNAi-treated oocytes resumed nuclear maturation at 8 h, and most oocytes had resumed by the end of the 24-h culture. Knockdown of Obox4 mRNA and loss of the OBOX4 protein by Obox4 RNAi was confirmed 4 h before GVBD occurred (Fig. 1A, B and C). The decreases in mRNA and protein are shown in Fig. 1B and C respectively, and are compared graphically in Fig. 1D. Obox4 loss induced GVBD despite the presence of oocytes in the IBMX-containing medium. This finding

Figure 1 Nuclear maturation of mouse oocytes occurred in an IBMX-supplemented medium after the transient knockdown of Obox4 mRNA and protein expression by Obox4 RNAi. (A) Nuclear maturation was measured after the Obox4 RNAi of oocytes cultured in an IBMX-supplemented medium for 24 h. Oocytes without GV or polar bodies were marked as GVBD. (B) Expression of Obox4 mRNA was eliminated within 4 h after Obox4 dsRNA injection. (C) Western blot analysis showed diminished levels of OBOX4 protein (100 oocytes per lane); actin was used as a loading control. (D) Bar graphs compare the changes shown in Fig. 1B and C. The experiment was performed three times, and the data are presented as the means ± S.E.M. Asterisks represent statistical significance at P<0.05.

Reproduction (2016) 151 369–378

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strongly suggests that Obox4 is necessary to maintain intact oocyte GV membranes.

**Premature activation of MPF/MAPK and STAT3 at the GV stage after Obox4 RNAi**

Because Obox4 knockdown induced GVBD resumption in IBMX-supplemented medium, changes in the activities of MPF and MAPK (which regulate oocyte maturation) were evaluated in oocytes arrested at the GV stage at 8 h. During spontaneous maturation in control oocytes, MPF and MAPKs were not activated at the GV (0 h) stage, and activation was observed after 2 h of in vitro maturation (Fig. 2A). Control oocytes that were incubated for 8 h in IBMX-supplemented M16 medium showed no activity (similar to oocytes at 0 h) (Fig. 2B: control, left three lanes). However, in Obox4-silenced GV oocytes after 8 h of incubation (lanes 1 through 17 in Fig. 2B), MPF and MAPK were activated even though the oocytes maintained the GV membrane in IBMX-supplemented M16 medium (Fig. 2B, C and D). Thus, the inappropriate untimely activation of MPF and MAPK in GV stage oocytes after Obox4 RNAi might initiate the GVBD of Obox4-silenced oocytes despite the presence of IBMX.

Total STAT3 amount and the phosphorylation of STAT3 at tyrosine 705 (pSTAT3, which activates STAT3) were assessed by western blot analysis. During spontaneous in vitro maturation, pSTAT3 increased at stage MI, and total STAT3 decreased (Fig. 2E). In Obox4 RNAi-treated GV-stage oocytes, significantly higher levels of pSTAT3 that were concurrent with decreased total levels of STAT3 were found at 8 h in IBMX-supplemented medium (Fig. 2F). This evidence suggests that Obox4 inhibits STAT3 phosphorylation to maintain an intact GV membrane. Obox4 might also play a role in initiating GVBD.

**The effect of the STAT3 activator on meiotic progression in IBMX-supplemented medium**

In the next step, we determined whether STAT3 activation is sufficient to drive oocyte nuclear maturation in IBMX-supplemented medium. As anticipated, the addition of IFNA, a STAT3 activator, to the IBMX-supplemented culture medium during the in vitro maturation of mouse oocytes stimulated meiotic progression concurrently with CC expansion (Fig. 3A).

![Figure 2](https://www.reproduction-online.org/)

**Figure 2** Obox4 RNAi-treated GV stage oocytes exhibited the premature activation of MPF, MAPK and STAT3. (A) MPF and MAPK activities during spontaneous in vitro oocyte maturation at 0, 2, 4, 6, 8 and 16 h in an IBMX-free medium, corresponding with stages GV, GVBD, pro-MII (4 and 6 h), MI, AI-TI and MII respectively. Each lane contained the lysate of one oocyte at the indicated stage. (B) Control (the first three lanes) and Obox4 RNAi-treated GV stage oocytes (17 lanes, 1–17) were collected at 8-h incubation in the IBMX-containing medium, and a dual kinase activity assay was performed. Each lane contained the lysate of one oocyte. (C) MPF activities of the oocytes 1–17 shown in Fig. 2B are summarized in bar graphs 1–17. (D) MAPK activities of the oocytes 1–17 shown in Fig. 2B are summarized in bar graphs 1–17. (E) Levels of STAT3 and pSTAT3 during oocyte meiotic maturation in vitro at 0 and 16 h, corresponding to stages GV and MII respectively. Proteins from 100 oocytes were loaded in each lane. (F) Levels of STAT3 and pSTAT3 in GV oocytes after microinjection with buffer (Control) and Obox4 dsRNA (Obox4 RNAi) and subsequent incubation for 8 h in IBMX-supplemented medium. The lysates of 100 oocytes were loaded in each lane. Obox4 RNAi-treated GV stage oocytes collected from 8-h culture after RNAi. The amounts of STAT3 and pSTAT3 protein are represented using bar graphs. The experiment was performed three times, and the data are presented as the means ± S.E.M. Asterisks represent statistical significance at P < 0.05.
When COCs were denuded and the nuclear maturation stage of oocytes was evaluated at 22 h of culture, all (100%) of the control oocytes were arrested at the GV stage in the presence of IBMX; in contrast, half (50.9%) of the IFNA-treated oocytes had resumed GVBD (Fig. 3B).

During spontaneous in vitro maturation, STAT3 activation via the phosphorylation of STAT3 (pSTAT3) increased at the MI stage, and total STAT3 decreased (Fig. 2E). Likewise, pSTAT3 levels were higher in GV oocytes that were incubated in vitro with IFNA for 22 h than in control GV oocytes (Fig. 3C).

To confirm CC expansion, changes in the expression of expansion markers (such as amphiregulin (Areg), prostaglandin-endoperoxide synthase 2 (Ptgs2), hyaluronic synthase 2 (Has2), pentraxin 3 (Ptx3) and TNFA-induced protein 6 (Tnfaip6)) were assessed. The expression of these genes in CCs was higher after treatment with the STAT3 activator IFNA than in the control group (Fig. 3D). We verified that each gene exhibited a clean and precise melting curve to confirm the purity of each gene product (Supplement 2, see section on supplementary data given at the end of this article).

We also measured the activities of MPF and MAPK in IFNA-treated GV stage oocytes to determine the dynamics of these kinases upon STAT3 activation. MPF and MAPK activities were increased (Fig. 3E), similar to the observations made in Obox4-silenced GV stage oocytes (Fig. 2B). Therefore, STAT3 activation is evidently involved in the Obox4 RNAi induction of the premature activation of MPF and MAPK at the GV stage; this process culminates in the resumption of GVBD despite the presence of IBMX in the medium.

Changes in the amount of pSTAT3 were assessed to evaluate its bona fide association with Obox4. A significantly higher level of pSTAT3 was found in Obox4 RNAi-treated GV stage oocytes at 8 h in IBMX-supplemented medium (Fig. 4A and B). To further confirm that the increased pSTAT3 levels were induced by Obox4 RNAi, we treated oocytes with 1 μM Statick and found that the addition of Statick to the IBMX-containing maturation medium inhibited STAT3 phosphorylation somewhat in Obox4-silenced GV oocytes. These results strongly support the fact that Obox4 plays a role as a suppressor of STAT3 activation and MPF/MAPK activation to maintain the intact GV membrane in oocytes.

**Figure 3** Addition of IFNA, a STAT3 activator, to the culture medium stimulated the resumption of oocyte meiotic maturation and cumulus expansion of the COCs. (A) Cumulus expansion occurred in vitro upon addition of the STAT3 activator IFNA. COCs were cultured in 100 μl M16 medium under mineral oil for 22 h. (B) Relative percentage of oocytes maintained at the GV stage. At 22 h of culture with IFNA, the oocytes were denuded of cumulus cells, and meiotic maturation status was measured. The experiment was performed three times, and the data are presented as means ± S.E.M. Asterisks represent significant differences compared to the control group (*P < 0.05*). (C) Levels of pSTAT3 and STAT3 in IFNA-treated GV stage oocytes that were collected at 22 h. Lysates of 100 oocytes were loaded in each lane. (D) Expression of cumulative expansion markers. The levels of Areg, Ptgs2, Has2, Ptx3 and Tnfaip6 mRNA in cumulus cells obtained from IFNA-treated COCs that contained GV stage oocytes were measured and normalized to the level of Gapdh in the same sample. (E) Dual kinase activity assay for MPF and MAPK to determine the effect of STAT3 activator treatment. Each lane represents the lysate of one oocyte. Control (three lanes) and STAT3 activator (IFNA)-treated GV stage oocytes (17 lanes) were collected at 22 h of culture. MPF and MAPK activities are summarized as bar graphs in the lower column; C, control oocytes; lanes number 1–17: 17 Obox4 RNAi-treated oocytes per lane. Red lines indicate relative kinase activities in control oocytes to that in 1.
Premature condensation of chromosomes in GV stage oocytes after Obox4 RNAi

The increased MPF/MAPK activity that was documented in Obox4-depleted oocytes raised the possibility that Obox4 might be a key factor in chromosome condensation or filament network organization during meiotic progression. Control GV stage oocytes that were sampled at 8 h in IBMX-supplemented medium exhibited chromosomes that appeared normal for the GV stage (Fig. 5A and B). However, as expected, Obox4-depleted GV stage oocytes contained aggregated chromosomes (white arrows) and aggregated tubulins (yellow arrows) which are not supposed to be aggregated at GV stage. These aggregated at incorrect timing had a structure that is very similar to that of microtubule-organizing center, adjacent to the inner facet of the GV membrane (Fig. 5C and D).

Discussion

In the present study, Obox4 was observed to act as an upstream regulator of MPF/MAPK and STAT3 activation, by which intact oocyte GVs undergo GVBD. Thus, Obox4 inhibits nuclear membrane breakdown in oocytes and plays a key role in maintaining the GV membrane in immature oocytes.

The major, important and exciting finding was that the activities of MPF and MAPK in mouse oocytes were prematurely activated at the GV stage under Obox4 deficiency. This is a new discovery regarding the upstream regulation of both MPF and MAPK in mouse oocytes. The MAPK cascade is important in regulating oocyte meiotic maturation and functions in parallel and/or in conjunction with MPF (Nebreda & Ferby 2000, Palmer & Nebreda 2000, Kotani & Yamashita 2002, Fan & Sun 2004). During normal oocyte maturation, MAPK is activated either after or simultaneously with MPF activation, depending on species. In mice, MAPK activation occurs ~2 h after MPF activation and GVBD resumption (Verlhac et al. 1994); this finding was confirmed using a dual kinase activity assay (Fig. 2A). In goat oocytes, detectable MAPK activation occurred approximately 8 h after cultivation, later than in mouse oocytes and later
than the MPF activation and GVBD resumption (Dedieu et al. 1996). MPF and MAPK activities are low in canine oocytes at the GV and GVBD stages but are significantly enhanced in stage MI and MII oocytes (Saint-Dizier et al. 2004).

MPF comprises two subunits: p34\(^{cdc2}\) and cyclin B. The p34\(^{cdc2}\) subunit is localized to the centrosome, kinetochore and spindle microtubules (Bailly et al. 1989, Riabowol et al. 1989, Rattner et al. 1990). Accordingly, MPF has been implicated in crucial aspects of meiotic maturation, such as nuclear disassembly, chromosome condensation, microfilament rearrangement and reorganization of the intermediate filament network (Morgan et al. 1989, Chou et al. 1990, Moreno & Nurse 1990, Peter et al. 1990). Moreover, MAPK is localized to the meiotic spindle, and its activity is considered critical for the regulation of microtubule organization and spindle assembly during oocyte maturation (Verlhac et al. 1993, Fellous et al. 1994, Goto et al. 2002). Therefore, the premature, inappropriate activation of these two important kinases by Obox4 downregulation aggravates cellular processes that are involved in oocyte nuclear maturation, causing the aggregation of chromosomes and microtubules in the Obox4 RNAi-treated oocytes. The premature activation of MPF and MAPK reached levels that were critical for invigorating chromosome condensation, spindle formation and nuclear membrane breakdown.

The other important finding was that STAT3 activation was prematurely stimulated at the GV stage under Obox4 deficiency. In a cell-specific manner, STAT3 plays an important role in the regulation of cell apoptosis, proliferation and migration (Akira 1999). The level of pSTAT3 increases during oocyte maturation in vitro, as measured using the Bio-Plex phosphoprotein immunoassay (Han et al. 2005). However, the regulatory functions of activated STAT3 in mammalian oocyte meiosis remain incompletely understood. Leptin induces STAT3 phosphorylation in MII oocytes (Matsuoka et al. 1999). Leptin receptors appear to increase during oocyte maturation, and leptin activity is dependent on STAT3 phosphorylation (Matsuoka et al. 1999). In 2010, leptin-induced nuclear maturation of rabbit oocytes through the JAK2/STAT3 and MEK 1/2 pathways was reported (Arias-Alvarez et al. 2010). Here, we report that Obox4 is an upstream inhibitory regulator of STAT3 activation in GV oocytes. However, whether such outcomes reflect the direct effects of Obox4 depletion alone or reflect mutual interactions between STAT3 activation and MPF/MAPK activation remains to be determined (Fig. 6).

STAT3 is one of many substrates of extracellular regulated kinase (ERK; Chen et al. 2001). Numerous proteins in all cellular compartments are substrates of activated ERK including membrane proteins, cytoskeletal proteins and nuclear proteins (such as STAT3). In addition to this kinase-substrate relationship, links between STAT3 activation and MAPK activation, especially those that occur through the phosphorylation of ERK at tyrosine 705 (the position studied here), have been reported in various cell systems including bone marrow cells (Kamezaki et al. 2005) and cancer cells (Camporeale et al. 2014). Enhanced basal ERK phosphorylation was observed in STAT3-deficient bone marrow cells, indicating that STAT3 negatively regulates ERK activation in bone marrow cells (Kamezaki et al. 2005). The relationships between STAT3 phosphorylation and MAPK phosphorylation in regulating cancer cellular energy metabolism are strongly interrelated (Camporeale et al. 2014). Therefore, we postulate that the increased phosphorylation of MAP/MAPK and the phosphorylation of STAT3 after downregulation of the inhibitory regulator Obox4 can mutually interact

![Figure 6](image_url)  
Schematic presentation of the proposed role of Obox4 in maintaining the intact GV membrane and nuclear membrane breakdown (GVBD) of the oocytes. On a molecular level, Obox4 is an upstream negative regulator of STAT3 phosphorylation and MPF/MAPK activation in mouse oocytes. With transient knockdown of Obox4 expression, increased STAT3 phosphorylation and MPF/MAPK activities were manifested prematurely in GV stage oocytes and appeared to induce GVBD, despite the unfavorable environment for oocyte nuclear maturation in IBMX-supplemented medium. Oocytes lacking Obox4 expression thus failed to maintain intact GV membranes in IBMX-supplemented medium, enabling the resumption of GVBD.
in oocytes. However, we were unable to determine which phosphorylation occurs first in this case.

In conclusion, Obox4 was found to be an important upstream negative regulator of MPF/MAPK phosphorylation and STAT3 phosphorylation. The premature phosphorylation of these important signaling proteins at the GV stage by Obox4 downregulation was found to induce the premature nuclear membrane breakdown of the GV membrane in the inhibitory environment of an IBMX-containing culture medium.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0078.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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