

# Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption

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Luteinizing hormone (LH) acts on ovarian follicles to reinitiate meiosis in prophase-arrested mammalian oocytes, and this has been proposed to occur by interruption of a meiosis-inhibitory signal that is transmitted through gap junctions into the oocyte from the somatic cells that surround it. To investigate this idea, we microinjected fluorescent tracers into live antral follicle-enclosed mouse oocytes, and we demonstrate for the first time that LH causes a decrease in the gap junction permeability between the somatic cells, prior to nuclear envelope breakdown (NEBD). The decreased permeability results from the MAP kinase-dependent phosphorylation of connexin 43 on serines 255, 262 and 279/282. We then tested whether the inhibition of gap junction communication was sufficient and necessary for the reinitiation of meiosis. Inhibitors that reduced gap junction permeability caused NEBD, but an inhibitor of MAP kinase activation that blocked gap junction closure in response to LH did not prevent NEBD. Thus, both MAP kinase-dependent gap junction closure and another redundant pathway function in parallel to ensure that meiosis resumes in response to LH.

## INTRODUCTION

The meiotic cell cycle in mammalian oocytes begins in the fetal ovary, and then pauses in prophase until luteinizing hormone (LH) from the pituitary releases the arrest (Eppig et al., 2004; Mehlmann, 2005a; Jones, 2008). LH acts on receptors on the mural granulosa cells in the outer region of the follicle that surrounds the oocyte, and the signal is conveyed inwards through the cumulus cells to the oocyte. By a pathway that is incompletely understood, LH signaling results in a fall in cAMP in the oocyte (Schultz et al., 1983; Sela-Abramovich et al., 2006), relieving the inhibition of cyclin dependent kinase 1 (Cdk1, also known as Cdc2; Cdc2a – Mouse Genome Informatics) in the oocyte, and allowing the prophase-to-metaphase transition to occur (see Jones, 2008).

The cAMP that is required to maintain prophase arrest is produced in the oocyte itself, by the constitutive activity of the orphan G<sub>s</sub>-linked receptor Gpr3 that activates adenylyl cyclase (Mehlmann et al., 2002; Horner et al., 2003; Kalinowski et al., 2004; Mehlmann et al., 2004; Mehlmann, 2005b; Freudzon et al., 2005; Ledent et al., 2005; Hinckley et al., 2005). If Gpr3, G<sub>s</sub> or adenylyl cyclase is absent or inhibited, cAMP decreases and meiosis resumes. Related G<sub>s</sub> and cAMP-dependent regulatory systems operate in oocytes of humans (DiLuigi et al., 2008), rats (Hinckley et al., 2005) and amphibians (see Gallo et al., 1995; Ríos-Cardona et al., 2008).

In mammals, contact of the mural granulosa cells with the cumulus-oocyte complex is also required to maintain arrest; removal of the cumulus-oocyte complex from the follicle (Pincus and

Enzmann, 1935; Edwards, 1965), or physical separation of these layers within the follicle (Racowsky and Baldwin, 1989), causes meiosis to resume. Gap junctions are required as well, as the application of gap junction inhibitors causes meiotic resumption (Piontkewitz and Dekel, 1993; Sela-Abramovich et al., 2006).

The somatic cells contribute to the maintenance of elevated cAMP in the oocyte, because cAMP decreases when the oocyte is isolated from the follicle (Törnell et al., 1990), and this may occur by way of gap junctions, as the application of gap junction inhibitors to the follicle decreases cAMP in the oocyte (Sela-Abramovich et al., 2006). Possibly the essential molecule entering the oocyte from the somatic cells is cAMP itself, adding to that generated by the Gpr3/G<sub>s</sub> system in the oocyte. Alternatively, an inhibitor of cAMP phosphodiesterase might diffuse into the oocyte from the mural cells (Törnell et al., 1991). It has been proposed that LH might cause the gap junctions in the path between the mural granulosa cells and the oocyte to close, thus preventing the passage of the meiosis-inhibitory molecule (Gilula et al., 1978; Larsen et al., 1987).

Gap junctions connect all cells of the follicle, but the connexins comprising the gap junctions differ in the somatic cells versus the oocyte. Connexin 43 (Cx43, or Gja1) is the primary connexin in the somatic cell junctions (see Beyer et al., 1989; Okuma et al., 1996; Tong et al., 2006). Connexin 45 and a small amount of connexin 37 (Cx37, or Gja4) are also present (Okuma et al., 1996; Alcoléa et al., 1999; Veitch et al., 2004; Simon et al., 2006), but their contribution to the overall coupling between the somatic cells appears to be minor compared with that of Cx43 (see Simon et al., 1997; Tong et al., 2006). By contrast, Cx37 is expressed by mouse oocytes and is found at the oocyte surface in oocyte-somatic cell gap junctions, with little if any contribution from Cx43 (Beyer et al., 1989; Simon et al., 1997; Kidder and Mhaw, 2002; Veitch et al., 2004; Gittens and Kidder, 2005; Li et al., 2007). The oocyte-somatic cell gap junctions are probably homotypic junctions composed of Cx37 on both sides of the junction (Veitch et al., 2004), with the somatic cells immediately adjacent to the oocyte expressing Cx37 and apparently targeting it

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differentially to processes that they extend across the zona pellucida (Veitch et al., 2004; Simon et al., 2006). Deletion of the gene encoding Cx37 eliminates gap junction communication at the oocyte surface, as well as gap junction plaques at the oocyte surface as seen by electron microscopy (Simon et al., 1997). Thus, Cx37 is essential for the junctions at the oocyte surface, although the possibility that another unidentified connexin is also required cannot be eliminated.

In studies of transport across the oocyte surface in cumulus-oocyte complexes isolated from follicles after LH receptor stimulation, gap junction permeability did not decrease before nuclear envelope breakdown (NEBD) (Gilula et al., 1978; Eppig, 1982; Racowsky and Satterlie, 1985). However, the possibility of a decrease in gap junction communication between the somatic cells, which could also result in the inhibition of a signal between the mural cells and oocyte (Larsen et al., 1987), was not investigated. In support of this concept, LH causes a rapid dispersion of the orderly packing pattern of the connexins in the membranes of the somatic cells of the follicle (Larsen et al., 1981), rapid phosphorylation of Cx43 (Granot and Dekel, 1994; Sela-Abramovich et al., 2005), and rapid closure of gap junctions between granulosa cells grown in culture (Sela-Abramovich et al., 2005; Sela-Abramovich et al., 2006). But whether it causes junction closure in intact ovarian follicles, and, if so, where and when relative to the time of meiotic resumption, is unknown. Thus, the possible role of gap junctions in the regulation of meiotic resumption in response to LH is unresolved.

To investigate these issues, we microinjected fluorescent tracers into intact follicle-enclosed mouse oocytes, and monitored their diffusion between the interconnected cells of the follicle by using two-photon microscopy and redistribution after photobleaching. We show that gap junction permeability between the somatic cells of the follicle decreases prior to NEBD, and establish that the decreased permeability results from MAP kinase-dependent phosphorylation of Cx43 on serines 255, 262 and 279/282. We then examine the functional relationship of these events to the reinitiation of meiosis, and show that although MAP kinase-dependent gap junction closure is one component of the mechanisms by which LH causes meiotic resumption, another signaling pathway also functions in parallel.

## MATERIALS AND METHODS

### Follicle culture

Antral follicles were dissected from the ovaries of 22- to 25-day-old B6SJL/F1 mice (Jackson Laboratory, Bar Harbor, ME), as approved by the University of Connecticut Animal Care Committee. They were cultured for 24–30 hours on Millicell culture plates (~12 follicles per plate; PICMORG50, Millipore, Billerica, MA), in MEM $\alpha$  (12000-022, Invitrogen, Carlsbad, CA) with 25 mM NaHCO<sub>3</sub>, 75  $\mu$ g/ml penicillin G, 50  $\mu$ g/ml streptomycin, 5% FCS (16000-044, Invitrogen), 10 ng/ml ovine follicle stimulating hormone [FSH, from A. F. Parlow (National Hormone and Peptide Program, Torrance, CA; NHPP)], and 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin and 5 ng/ml selenium (Sigma, St Louis, MO), equilibrated with 5% CO<sub>2</sub> and 95% air. Meiotic resumption was stimulated with 10  $\mu$ g/ml ovine LH (NHPP). The nucleus of the oocyte was visible within the follicle on the culture plate (see Norris et al., 2007), allowing the observation of NEBD. For a few experiments, we used preantral follicles (Fig. 6C), or antral follicles from mice that had been injected with 4 IU of pregnant mare serum gonadotropin (PMSG; NHPP) 42 hours prior to follicle isolation (see Fig. S2 in the supplementary material).

### Microinjection of follicle-enclosed oocytes

Antral follicles were placed in an injection chamber in which they were flattened between two coverslips spaced 200  $\mu$ m apart (250  $\mu$ m apart for antral follicles from PMSG-injected mice, and 100  $\mu$ m apart for preantral follicles), and quantitative microinjection of 10  $\mu$ l (5% of the 200  $\mu$ l volume of the oocyte) was carried out at 22°C (Jaffe and Terasaki, 2004; Norris et al., 2007; Jaffe et al., 2009).

### Gap junction tracers

Alexa Fluor 350 (A10439, Invitrogen;  $M_r$ =326) and Alexa Fluor 488 (A10436, Invitrogen;  $M_r$ =534) were dissolved in 100 mM NaCl, 5 mM PIPES, pH 6.8, with brief heating to ~90°C, and stored at -80°C. Alexa Fluor 350 was used at a stock concentration of 50 or 100 mM, resulting in an initial concentration in the oocyte of 2.5 or 5 mM; Alexa Fluor 488 was used at a stock concentration of 2 or 5 mM, resulting in an initial concentration in the oocyte of 100 or 250  $\mu$ M. Because of its smaller size, which results in greater permeability through the Cx37 channels at the oocyte surface (Weber et al., 2004), Alexa Fluor 350 was used, except where indicated.

### Two-photon imaging of Alexa Fluor 350

Two-photon microscopy allowed optimal visualization at an ~100  $\mu$ m depth within the follicle (Helmchen and Denk, 2005). Follicles were imaged in the coverslip chamber in which they had been injected with Alexa Fluor 350. We used a Zeiss LSM 510 system, with a Ti:Sapphire laser (Chameleon; Coherent, Santa Clara, CA) tuned to 720 or 740 nm, and a 20 $\times$ /0.8 NA objective. The non-descanned emitted light was collected through a 435–485 nm filter. Images were collected at the oocyte equator, using four different laser intensities to avoid saturation or too low a signal in all regions. The microscope stage was maintained at 37°C, with humidified 5% CO<sub>2</sub>/air.

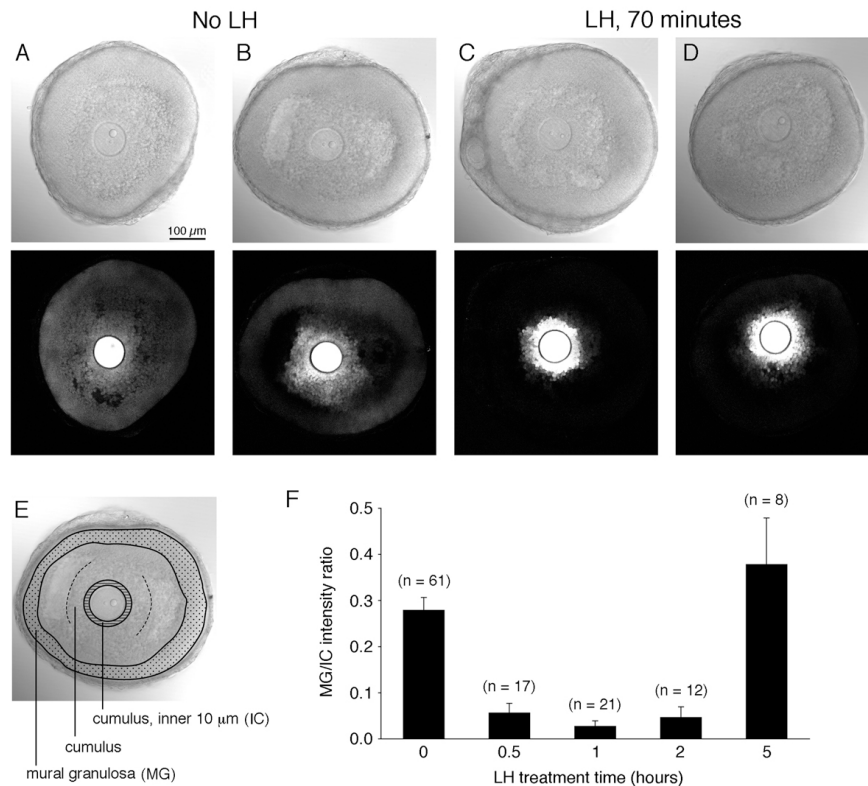
To quantify Alexa Fluor 350 fluorescence ratios in the mural granulosa/inner cumulus regions, the mural region was identified from a scanning transmission image, and the inner cumulus was defined as the region between the outer edge of the zona pellucida and a circle 10  $\mu$ m beyond the edge of the zona; this included the inner quarter-to-half of the cumulus mass (Fig. 1E). Autofluorescence determined from corresponding regions of uninjected follicles was subtracted. Measurements from images taken at different percent laser transmissions were normalized before calculating a ratio, using empirically determined conversion factors. For example, the specimen intensity increased threefold in changing from a 5 to a 10% laser transmission.

To compare Alexa Fluor 350 efflux from follicle-enclosed oocytes  $\pm$ LH, we recorded images at two to three time points between 9 and 22 minutes after injection, and calculated the percent decrease in oocyte intensity between 12 and 20 minutes. For this approach to be valid, there should be a large concentration gradient between the oocyte and the cumulus cells to minimize the effect of Alexa Fluor 350 diffusing back from the cumulus cells to the oocyte. At 20 minutes after injection, the concentration of Alexa Fluor 350 in the oocyte was still three to 14 times that in the inner cumulus cells (see Fig. 1A–D); the oocyte/inner cumulus cell concentration gradient was  $6.8 \pm 0.9$  (mean  $\pm$  s.e.m.,  $n=14$  follicles) for follicles without LH, and  $5.8 \pm 0.7$  ( $n=15$ ) for follicles exposed to LH for ~1 hour.

### Fluorescence redistribution after photobleaching of Alexa Fluor 488

Follicle-enclosed oocytes were injected with Alexa Fluor 488 and incubated on Millicell plates for 1–4 hours to allow the tracer to spread throughout the follicle. After exposure to LH, the follicles were placed in a coverslip chamber for photobleaching using a Zeiss LSM 510 microscope. We used Alexa Fluor 488, despite its lower permeability through the Cx37 channel compared with Alexa Fluor 350 (Weber et al., 2004), because its higher quantum yield and longer excitation wavelength reduce damage during photobleaching (Galbraith and Terasaki, 2003), which occurred during initial attempts with Alexa Fluor 350.

Using a 40 $\times$ /1.2 NA water immersion objective, and the 488 and 514 nm lines of a 30 milliwatt Argon laser at 100% power, we photobleached a 60 $\times$ 20  $\mu$ m rectangle in the mural granulosa cell layer, ~20  $\mu$ m below the follicle surface. A 4.5-second exposure decreased the fluorescence intensity by ~50%. Post-bleach images were collected using the same objective, but with the laser intensity reduced to 0.5% power, using a 505 nm long pass filter and the confocal pinhole fully open; images were collected at 1.6-second intervals for 1 minute, and then at 10-second intervals for 2–5 minutes. These monitoring conditions did not significantly bleach the Alexa Fluor 488. To compare the time course of fluorescence redistribution with



**Fig. 1. LH reduces gap junction permeability between cumulus and mural granulosa cells.** Determined by injecting Alexa Fluor 350 into the oocyte and imaging its diffusion within the follicle at 20 minutes after injection. (A,B) No LH. (C,D) LH applied 70 minutes before injecting Alexa Fluor 350. Upper panels show scanning transmission images; lower panels show Alexa Fluor 350 distribution. In A and B, Alexa Fluor 350 diffused throughout the mural granulosa cells, whereas in C and D, the Alexa Fluor 350 spread into the cumulus cells, but little or none was seen in the mural granulosa cells. (E) Regions of the follicle used for the measurements shown in F. The dashed lines indicate the approximate border between the cumulus mass and the antral space. (F) Ratio of fluorescence intensity in the mural granulosa cells to that in the inner cumulus cells, as a function of LH treatment time. Measurements were made at 20 minutes after injecting Alexa Fluor 350 into the oocyte. Bars show mean  $\pm$  s.e.m., and the numbers in parentheses indicate the number of follicles tested at each time point (0.5 hour=33–40 minutes, 1 hour=64–75 minutes, 2 hours=124–140 minutes, 5 hours=4.5–5.7 hours).

and without LH, we measured the change in Alexa Fluor 488 intensity in the bleached region during the first minute (between 5 and 65 seconds) after the end of the bleach.

### Antibodies

Mouse anti-Cx43 antibodies, Cx43IF1 made against amino acids 360–382, and Cx43NT1 made against amino acids 1–20 (Cooper and Lampe, 2002; Lampe et al., 2006; Sosinsky et al., 2007), were prepared at the Fred Hutchinson Cancer Research Center Hybridoma Development Facility (Seattle, WA). Phosphospecific antibodies for pERK (pMAPK; sc-7383) and Cx43 phosphorylated at S255 (sc-12899) and S262 (sc-17219-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against pS279/S282-Cx43, pS368-Cx43, pY247-Cx43 and pY265-Cx43 were custom prepared, affinity purified, and tested for specificity as previously described (Solan et al., 2007; Solan and Lampe, 2008). The antibody against vinculin was obtained from Sigma (V4505). Two independent Cx37 antibodies were made in rabbits against a GST fusion protein (amino acids 229–333 of rat Cx37), and were affinity purified (Goliger and Paul, 1994; Simon et al., 2006).

### Immunoblotting

Samples for immunoblotting were prepared by washing the follicles in PBS and sonicating them in Laemmli sample buffer containing 5%  $\beta$ -mercaptoethanol, 10 mM NaF, 1 mM Na orthovanadate, 1 mM Pefabloc (Roche Applied Science, Indianapolis) and Roche Complete protease inhibitor cocktail. Each follicle contained  $\sim 3.5 \mu\text{g}$  of protein;  $5 \mu\text{g}$  of protein was loaded per lane.

Blots of follicles were probed with various rabbit Cx43 phosphospecific antibodies, applied together with a mouse monoclonal antibody recognizing total Cx43 (NT1). Primary antibodies were used at 0.2–0.7  $\mu\text{g}/\text{ml}$ . The rabbit phosphospecific antibodies were detected with IRDye800-labeled anti-rabbit IgG (611–731–127, Rockland Immunochemicals, Gilbertsville, PA) and the monoclonal NT1 with Alexa Fluor 680 goat anti-mouse IgG (A21058, Invitrogen). Binding of the two secondary antibodies was simultaneously quantified by using the LI-COR Biosciences Odyssey infrared imaging system and associated software (Lincoln, NE). Images

were converted from 16 bits to 8 bits, after maximizing the dynamic range of pixel intensity using the ‘levels’ function in Adobe Photoshop. Blots of isolated oocytes probed with the Cx37 antibody ( $\sim 0.3 \mu\text{g}/\text{ml}$  IgG) were visualized with an HRP-conjugated secondary antibody (sc-2030, Santa Cruz Biotechnology) and ECL Plus reagents (GE Healthcare, Piscataway, NJ).

### Immunofluorescence microscopy

For total Cx43 immunofluorescence, follicles were fixed with 4% paraformaldehyde, and embedded and frozen (Norris et al., 2007). For pS279/S282 immunofluorescence, follicles were frozen without fixation, in gelatin capsules containing tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC). Cryosections (10  $\mu\text{m}$ ) were fixed with 50% MeOH/50% acetone at  $-20^\circ\text{C}$  for 1–2 hours, and then probed with the IF1 antibody (total Cx43, 1  $\mu\text{g}/\text{ml}$ ) and Alexa Fluor 488 goat anti-mouse IgG (A11029, Invitrogen), or with the pS279/S282 antibody (0.5  $\mu\text{g}/\text{ml}$  in a buffer containing 0.25% Tween-20) and Alexa Fluor 488 goat anti-rabbit IgG (A11034, Invitrogen). NaF (10 mM) and Na orthovanadate (500  $\mu\text{M}$ ) were included in the fixation and processing solutions. Sections were imaged using a  $40\times/1.2$  NA water immersion objective on a Zeiss LSM 510 or Pascal confocal microscope.

### U0126, carbenoxolone

U0126 and an inactive analog, U0124, were obtained from EMD Chemicals (La Jolla, CA), dissolved in DMSO at 100 mM, and diluted to 10 or 100  $\mu\text{M}$  for use. Follicles were pre-incubated with U0126 for 1 hour before addition of LH. Carbenoxolone was obtained from Sigma.

## RESULTS

### LH causes a rapid, but transient, decrease in the permeability of gap junctions between the somatic cells of the ovarian follicle

To investigate the effect of LH on gap junction permeability, we injected antral follicle-enclosed mouse oocytes with a gap junction permeant fluorescent molecule, Alexa Fluor 350, and monitored its



diffusion into the cumulus and mural granulosa cells, using two-photon microscopy (Fig. 1). Except where indicated, we used 320- to 360- $\mu$ m diameter follicles that had been isolated from prepubertal mice. To obtain LH responsiveness, the follicles were cultured with FSH for 24-30 hours *in vitro*.

In most follicles that had not been exposed to LH, Alexa Fluor 350 had spread into the cumulus cells and all of the mural granulosa cells by 10-20 minutes after injection into the oocyte (Fig. 1A,B). The tracer spread outwards into the mural granulosa cells from the site of contact with the cumulus cells (see Fig. S1 and Fig. S2A in the supplementary material). By contrast, in most follicles that had been exposed to LH for 0.5-2 hours prior to injection, Alexa Fluor 350 was present almost exclusively in the oocyte and cumulus cells, or in the oocyte and cumulus cells with a small amount of local diffusion into the mural cells at the cumulus/mural cell border (Fig. 1C,D). The decrease in gap junction permeability preceded NEBD, which begins at  $\sim$ 2 hours after application of LH (see Fig. 7C). The decrease was transient, as follicles that had been exposed to LH for  $\sim$ 5 hours before injecting Alexa Fluor 350 showed tracer diffusion throughout the mural granulosa cells (see Fig. S2 in the supplementary material).

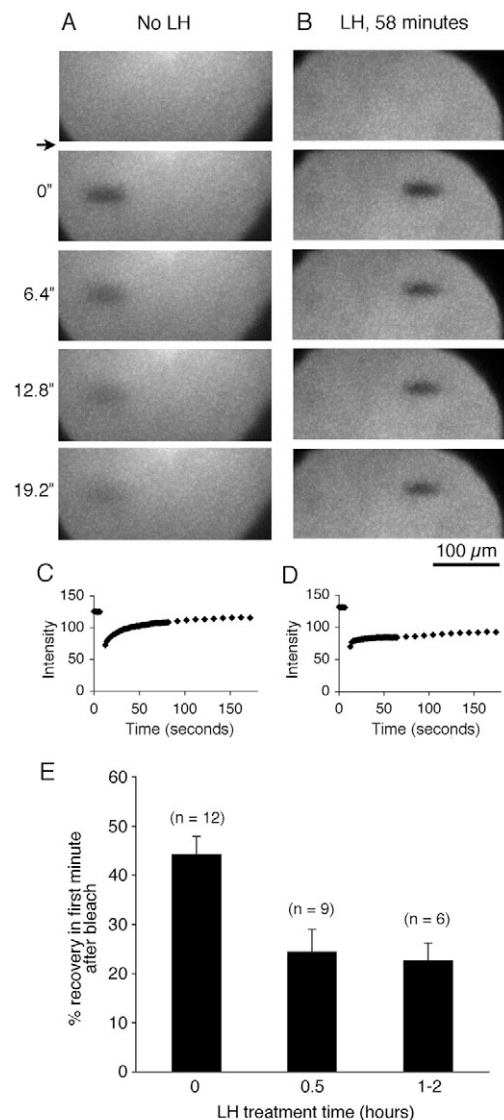
A similar transient decrease in gap junction permeability in response to LH was seen in follicles from prepubertal mice that had been injected with PMSG to stimulate follicle growth and LH receptor development *in vivo* (see Fig. S2 in the supplementary material). However, owing to the optical density of these  $\sim$ 500- $\mu$ m diameter follicles, which made them difficult to inject and image, we used the optically clearer follicles described above for all further studies.

Following LH exposure, Alexa Fluor 350 was mostly restricted to the two to three layers of cumulus cells closest to the oocyte (Fig. 1C,D; see also Fig. S2 in the supplementary material). These cells are directly connected to the oocyte by processes that extend through the intervening cumulus cells and zona pellucida to form gap junctions at the oocyte surface (Anderson et al., 1978). Thus, the restriction of Alexa Fluor 350 to the inner cumulus cells of LH-stimulated follicles is most likely to indicate diffusion through the Cx37 channels that comprise the gap junctions at the oocyte surface, but not through the Cx43 channels that are predominant throughout the somatic cells (see Introduction).

To quantify the LH-induced changes in gap junction permeability, we measured the ratio of the average fluorescence intensity in the mural granulosa cells to that in the inner cumulus cells, at 20 minutes after injection of Alexa Fluor 350 (Fig. 1E). For follicles treated with LH for 0.5-2 hours, this ratio was less than for follicles without LH treatment. By 5 hours after application of LH, the ratio had returned to the pre-LH level (Fig. 1F).

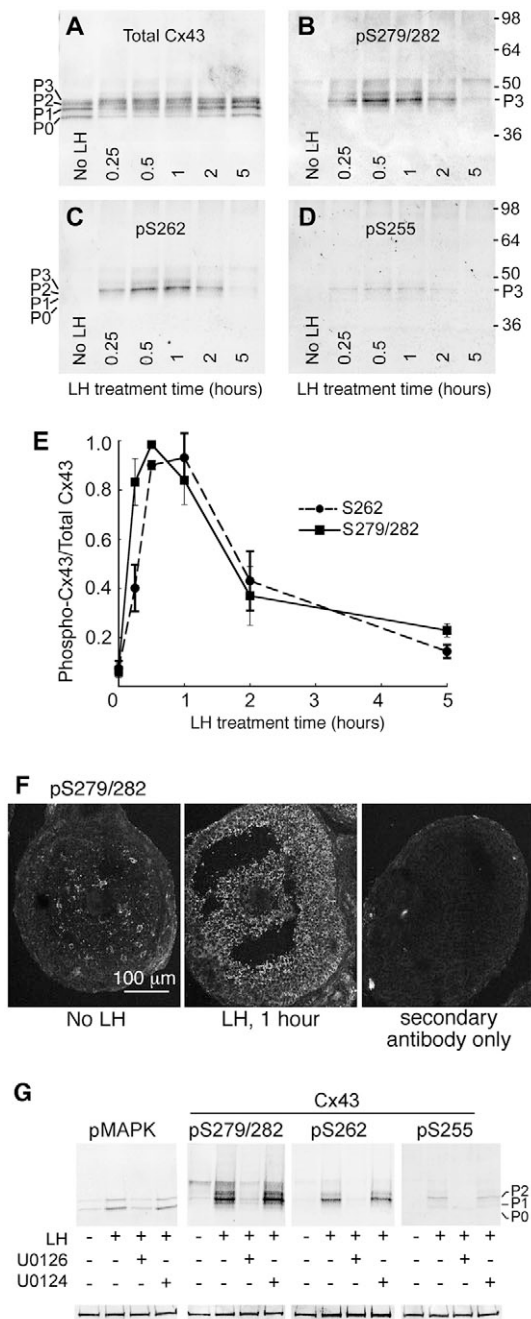
### The LH-induced permeability decrease also occurs in the junctions between mural granulosa cells

The barrier to small molecule transfer that is established between the cumulus and mural granulosa cells could result from gap junction closure only within this region, or from a general closure of gap junctions throughout the somatic cell layers. To investigate if LH caused gap junctions to close between the mural granulosa cells, we loaded the cells of the follicle with the fluorescent tracer Alexa Fluor 488, photobleached a region within the mural granulosa cell layer, and monitored the redistribution of fluorescence in the bleached region (Fig. 2). At one minute after the bleach, the fluorescence intensity in most control follicles had recovered to 40-50% of its pre-bleach value (Fig. 2A,C,E). By contrast, in almost all follicles that had been exposed to LH for 0.5-



**Fig. 2. LH reduces gap junction permeability between mural granulosa cells.** Determined by fluorescence redistribution after photobleaching of Alexa Fluor 488. (A) No LH. (B) LH applied 58 minutes before photobleaching. For A and B, a rectangular region was photobleached for 4.5 seconds, between the images indicated by the arrow. Each column shows images before and at various times (in seconds) after the end of the bleach. (C,D) Fluorescence intensity in the bleached region as a function of time, for the images shown in A and B. (E) Percent recovery of fluorescence intensity in the bleached region during the first minute after the bleach, as a function of LH treatment time. Bars show mean  $\pm$  s.e.m., and the numbers in parentheses indicate the number of follicles tested at each time point (0.5 hour=27-40 minutes, 1-2 hours=58-120 minutes).

2 hours, the fluorescence intensity returned more slowly, indicating that LH had caused a decrease in gap junction permeability throughout the mural granulosa cell layer (Fig. 2B,D,E). We were unable to use photobleaching to investigate gap junction permeability within the cumulus cell layer, or between cumulus cells and the oocyte, because these regions were too deep within the tissue to bleach effectively (see Fig. S3 in the supplementary material).



**Fig. 3. LH causes MAP kinase-dependent phosphorylation of multiple serines of Cx43.** (A–D) Immunoblots of follicles,  $\pm$ LH for various times, probed for total Cx43, and for phosphorylation on particular serines as indicated. The fastest migrating species, marked P0, contains the unphosphorylated form, and P1, P2 and P3 are three different commonly observed phosphorylated forms. (E) Densitometric analysis of the relative amount of phosphorylation on S262 and S279/S282 of Cx43, as a function of time after LH addition. The results are expressed as the ratio of the density of the phosphorylated bands divided by the density of the bands representing total Cx43 from the same blot, and are normalized to the maximum value obtained (at 0.5 or 1 hour) for the time series. The results of four independent experiments for each antibody were combined (mean  $\pm$  s.e.m.). A similar time course was seen in three independent experiments with the pS255 antibody, but the signal was too low to allow meaningful quantitation. (F) Immunofluorescence images of pS279/S282 Cx43 in antral follicles with or without a 1 hour exposure to LH. The images are representative of six follicles without LH, and 11 follicles with LH. (G) Immunoblots of follicles with or without a 1 hour exposure to LH, in the presence of 10  $\mu$ M of the MEK inhibitor U0126 or its inactive analog U0124. The blots were probed for phosphorylation of MAP kinase and particular serines of Cx43, and also for vinculin (lower row), to confirm that protein amounts in each lane were equivalent. Similar results were obtained in three independent experiments.

### A decrease in the amount or localization of Cx43 protein does not account for the permeability decrease

Immunoblots of Cx43 in follicles without LH, or which had been exposed to LH for 0.25, 0.5, 1, 2, or 5 hours, all showed approximately the same amount of Cx43 protein (Fig. 3A). Multiple Cx43 bands were seen, representing multiple phosphorylation states (see below), but the total density of these bands varied by <20% over this time course (analysis of six similar blots). At 1 hour after the application of LH, the localization of Cx43, as detected by confocal imaging of immunofluorescence, was unchanged (Fig. 4). Thus, the LH-induced decrease in gap junction communication detected at 1 hour after LH application is most likely to be caused by closure of the Cx43 channels.

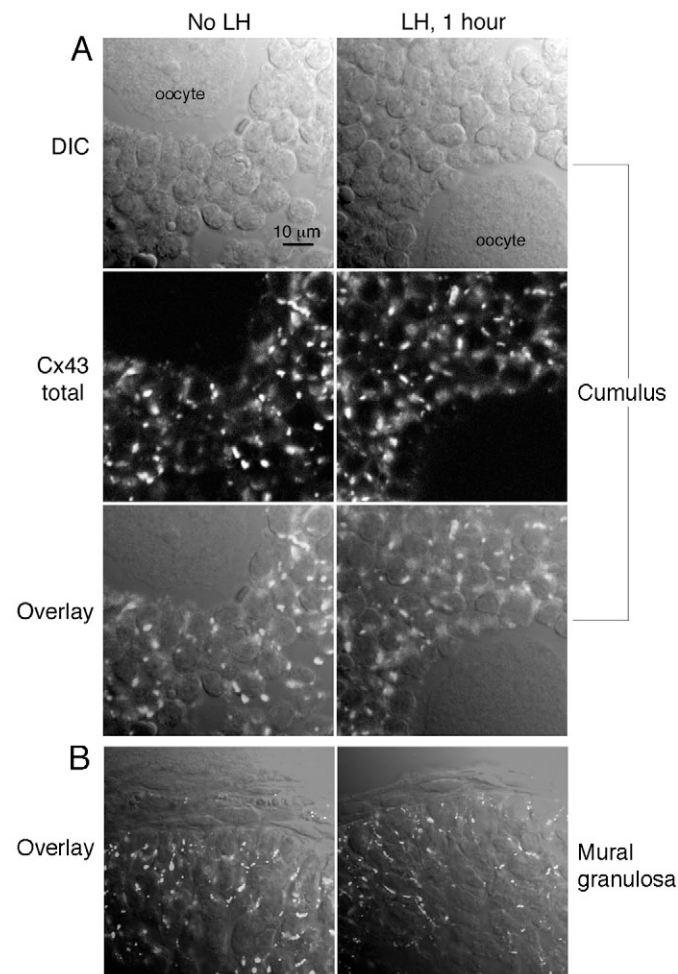
### LH causes phosphorylation of Cx43 on several regulatory serines

LH application to follicles causes a shift in the SDS-PAGE mobility of Cx43, which is due to phosphorylation on unspecified sites (Kalma et al., 2004) (Fig. 3A). To determine whether known regulatory sites on Cx43 were phosphorylated in response to LH application, we labeled blots of follicle proteins with antibodies that recognize particular phosphorylated serines or tyrosines of Cx43. Serines 255, 279, 282 and 368, and tyrosines 247 and 265, were of particular interest, because phosphorylation on these sites is required for the closure of gap junction channels by MAP kinase (S255/S279/S282) (Warn-Cramer et al., 1998), by PKC (S368) (Lampe et al., 2000), and by Src family kinases (Y247/Y265) (Swenson et al., 1990; Lin et al., 2001). Phosphorylation on S262 is also associated with a decreased permeability of Cx43 gap junctions (Doble et al., 2004).

Immunoblots using three antibodies specific for phosphoserines 279/282, 262 and 255 of Cx43 showed little phosphorylation on these sites in follicles that had not been exposed to LH (Fig. 3B–D,G), but by 15 minutes after the application of LH, phosphorylation

### LH does not cause a detectable decrease in the permeability of the gap junctions between the oocyte and cumulus cells

Images like those shown in Fig. 1 did not show an obvious effect of LH on the permeability of gap junctions at the oocyte surface, and measurements of the percentage decrease in fluorescence intensity in the oocyte between 12 and 20 minutes after Alexa Fluor 350 injection did not show a significant difference with or without an  $\sim$ 1 hour exposure to LH [without LH,  $33 \pm 5\%$  (mean  $\pm$  s.e.m.),  $n=14$  follicles; with LH,  $26 \pm 3\%$ ,  $n=15$ ]. Thus, although a small change might have been missed by this measurement method, gap junction permeability at the oocyte surface did not show a major decrease like that occurring in the somatic cells.



**Fig. 4. Immunofluorescence of Cx43 in antral follicles with or without a 1 hour exposure to LH.** (A) Cumulus-oocyte region. Upper panels show scanning transmission differential interference contrast (DIC) images, middle panels show total Cx43 fluorescence, and lower panels show overlayed images. (B) Mural granulosa region, showing overlayed images as in A. The images are representative of eight follicles without LH, and seven with LH; both the cumulus and the mural granulosa regions were examined in each follicle.

on each of these sites increased. Phosphorylation was maximal at 0.5–1 hour after LH application, and then decreased between 2 and 5 hours; at 5 hours, the level of phosphorylation was only slightly greater than that before LH exposure (Fig. 3B–E). This time course of Cx43 phosphorylation, and subsequent dephosphorylation, paralleled the time course of changes in gap junction permeability (Fig. 1). Like the decrease in gap junction permeability, phosphorylation on S279/S282 occurred in both the mural granulosa and the cumulus cells (Fig. 3F).

Immunoblots of follicles using three antibodies specific for phosphorylation at Y247, Y265 and S368 of Cx43 (Solan et al., 2007; Solan and Lampe, 2008) showed little or no signal, and no change in response to LH, whereas robust signals were seen in positive control lanes with comparable amounts of Cx43 protein from LA-25 cells expressing active v-Src, or NRK cells treated with phorbol ester (see Fig. S4 in the supplementary material). This indicated that the LH-induced decrease in Cx43 permeability was not due to phosphorylation at these sites.

### LH-stimulated Cx43 phosphorylation is MAP kinase dependent

Because S279/S282 and S255 of Cx43 are known MAP kinase substrates (Warn-Cramer et al., 1996; Warn-Cramer et al., 1998), and because LH activates MAP kinase in the follicle (Su et al., 2002; Kalma et al., 2004; Panigone et al., 2008), we used the MEK-specific inhibitor U0126 (Favata et al., 1998) to test whether the LH-induced phosphorylation of Cx43 was MAP kinase dependent. U0126 (10  $\mu$ M), which inhibited the LH-induced increase in phosphorylation of MAP kinase, also inhibited phosphorylation on Cx43 S279/S282, S262 and S255 (Fig. 3G). These results are consistent with previous gel shift evidence for MAP kinase dependence of the LH-stimulated phosphorylation of Cx43 in rat follicles (Sela-Abramovich et al., 2005).

### Inhibition of gap junction permeability is sufficient to cause meiotic resumption

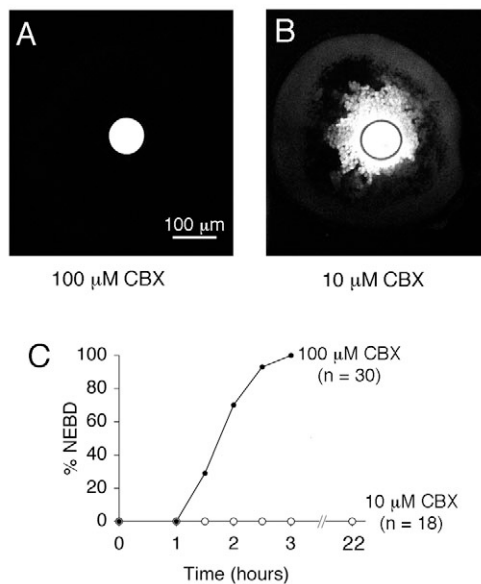
The experiments described above provide the first direct evidence that LH action on the intact follicle decreases gap junction permeability between the somatic cells, prior to NEBD, and that this is linked to the MAP kinase-dependent phosphorylation of Cx43 on multiple serine residues. The closure of the junctions isolates the inner cumulus-oocyte complex from signals that pass through the junctions from the mural granulosa cells. Because mechanical isolation of the cumulus-oocyte complex is sufficient to cause meiotic resumption (Pincus and Enzmann, 1935; Racowsky and Baldwin, 1989), we examined whether the inhibition of gap junction communication between the mural granulosa cells and the oocyte would cause meiotic resumption.

In the absence of a method to rapidly and selectively close only the Cx43 channels, as occurs in response to LH, we examined the effect of applying the general gap junction inhibitor carbenoxolone (CBX) (Rozental et al., 2001). A previous study had shown that 100  $\mu$ M CBX inhibits gap junction permeability between rat granulosa cells in culture (Sela-Abramovich et al., 2006), and, likewise, we found that 100  $\mu$ M CBX blocked gap junction communication between the somatic cells and the oocyte in intact mouse follicles (Fig. 5A). At a concentration of 10  $\mu$ M, CBX only partially inhibited gap junctional communication (Fig. 5B). In rat follicles, 100  $\mu$ M CBX has been found to cause NEBD, as assayed at 5 hours after CBX application (Sela-Abramovich et al., 2006). Similarly, we found that 100  $\mu$ M CBX caused NEBD in mouse follicles, and determined that, in most follicles, this occurred after 1–2 hours (Fig. 5C). A concentration of 10  $\mu$ M CBX did not cause NEBD (Fig. 5C).

We also used an antibody against the C-terminal cytoplasmic domain of Cx37 (Fig. 6A) to decrease gap junction communication between the cumulus cells and the oocyte, and thus indirectly to decrease communication between the mural cells and the oocyte. Injection of this antibody into follicle-enclosed oocytes decreased Alexa Fluor 350 diffusion from the oocyte (Fig. 6B), and the inhibition developed over a period of several hours (Fig. 6C). This suggested an effect on Cx37 turnover (Laird, 2006), which could decrease the number of channels in the plasma membrane, rather than an effect on individual channel permeability. Corresponding to the reduction in gap junction communication, NEBD occurred at 6–12 hours after injection of the antibody (Fig. 6D).

These two different ways of reducing gap junction communication in the follicle both resulted in meiotic resumption, supporting the conclusion that the signal between the mural granulosa cells and the oocyte that maintains meiotic arrest is





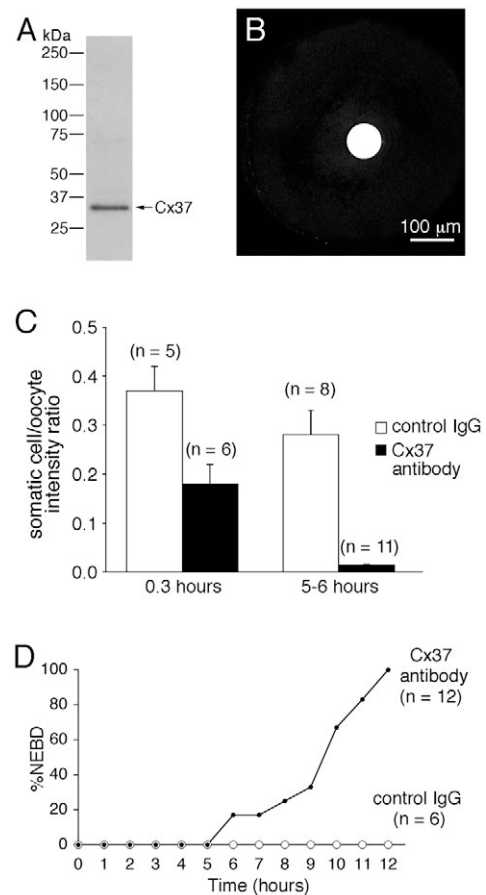
**Fig. 5. Carbenoxolone decreases gap junction permeability and causes meiotic resumption.** (A) CBX at a concentration of 100  $\mu$ M reduces gap junction permeability to an undetectable level. (B) CBX at a concentration of 10  $\mu$ M reduces gap junction permeability only partially. For A and B, Alexa Fluor 350 was injected into follicle-enclosed oocytes at 58–70 minutes after applying CBX, and follicles were imaged 20 minutes later. A and B are representative of the results of injections into six and 11 follicle-enclosed oocytes, respectively. See Fig. 1A,B for comparison without CBX. (C) A concentration of 100  $\mu$ M CBX causes NEBD, but 10  $\mu$ M CBX does not.

conveyed by way of gap junctions. Thus, the junction closure that occurs in response to LH would have the consequence of releasing the inhibition.

### LH also activates a meiosis-stimulatory pathway that is independent of MAP kinase-mediated gap junction closure

As noted above, 10  $\mu$ M of the MEK inhibitor U0126 inhibited Cx43 phosphorylation (Fig. 3G). U0126 (10  $\mu$ M) also inhibited gap junction closure in response to LH (Fig. 7A). Based on the ratio of fluorescence in the mural granulosa/inner cumulus cells at 20 minutes after injecting Alexa Fluor 350 into the oocyte, the permeability of the Cx43 channels in follicles that had been exposed to LH for 66–72 minutes in the presence of 10  $\mu$ M U0126 was indistinguishable from that in follicles that had not been exposed to LH (Fig. 7B). Thus, although we cannot eliminate the possibility of a change in channel properties that was not detectable by this tracer, it appears that 10  $\mu$ M U0126 effectively reverses the decrease in channel permeability caused by LH treatment.

However, as reported in a previous study of mouse follicles (Su et al., 2003), 10  $\mu$ M U0126 caused little, if any, decrease in the percentage of oocytes undergoing NEBD in response to LH, with inhibition seen only when the U0126 concentration was increased to 100  $\mu$ M (Fig. 7C) (see Su et al., 2003). U0126 treatment at a concentration of 10  $\mu$ M also caused no significant delay in the time course of NEBD (Fig. 7C). Thus, LH stimulated NEBD even under conditions where gap junction closure was inhibited. This finding supports the conclusion that although gap junction closure is sufficient to cause meiotic resumption (Sela-Abramovich et al.,

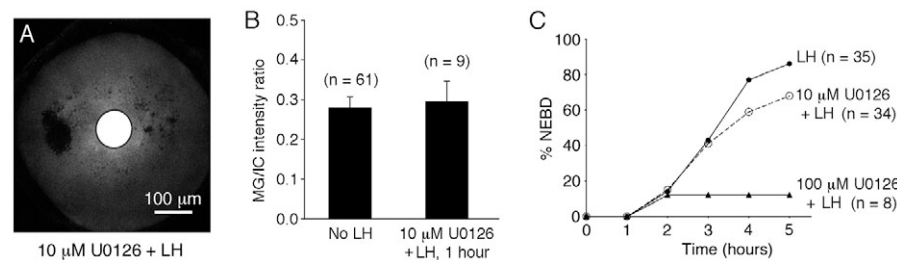


**Fig. 6. Injection of follicle-enclosed oocytes with an antibody against Cx37 decreases gap junction communication and causes meiotic resumption.** (A) Immunoblot of mouse oocytes (1  $\mu$ g of protein), showing the specificity of the Cx37 antibody. (B) An antral follicle in which the oocyte was injected with 2  $\mu$ M of the Cx37 antibody, and then, after 5 hours, injected with Alexa Fluor 350; the follicle was imaged 20 minutes later. B is representative of the results obtained with three antral follicle-enclosed oocytes. (C) Diffusion of Alexa Fluor 350 from the oocyte to the somatic cells is inhibited only weakly when tested at 20 minutes after the Cx37 antibody injection, but is inhibited strongly at 5–6 hours. The experiments shown in C were carried out using 140–180  $\mu$ m diameter preantral follicles, because these provided a technically easier system for investigating multiple conditions. Somatic cell/oocyte intensity ratios were measured at 30 minutes after injection of the Alexa Fluor 350; bars show mean  $\pm$  s.e.m.; *n*, number of follicles. (D) Injection of the Cx37 antibody causes NEBD, whereas a control IgG has no effect.

2006) (Figs 5, 6), LH also activates an additional meiosis-stimulatory pathway that does not require the MAP kinase-dependent closure of gap junctions.

### DISCUSSION

The results described here establish that LH causes rapid MAP kinase-dependent phosphorylation and closure of the gap junctions between somatic cells of the mouse ovarian follicle. The Cx43 junctions throughout the somatic cell compartment close, whereas the Cx37 junctions with the oocyte remain open (Fig. 8). The net effect is that a barrier to diffusion is established between the mural granulosa cells and the oocyte. The presence of the barrier is



**Fig. 7. Inhibition of MAP kinase activation prevents gap junction closure, but does not inhibit nuclear envelope breakdown in response to LH.** (A) U0126 (10  $\mu$ M) inhibits LH-stimulated gap junction closure. Alexa Fluor 350 was injected at 72 minutes after applying LH, and the follicle was imaged 20 minutes later. (B) Ratio of fluorescence intensity in the mural granulosa cells to that in the inner cumulus cells, comparing follicles that had been treated with LH for 1 hour (66–72 minutes) in the presence of 10  $\mu$ M U0126, and follicles without LH treatment (data from Fig. 1F). Measurements were made at 20 minutes after injecting Alexa Fluor 350 into the oocyte. Bars show mean  $\pm$  s.e.m. (C) A concentration of 100  $\mu$ M U0126 inhibits LH-stimulated NEBD, but 10  $\mu$ M U0126 does not. For B and C, *n* is the number of follicles tested for each condition.

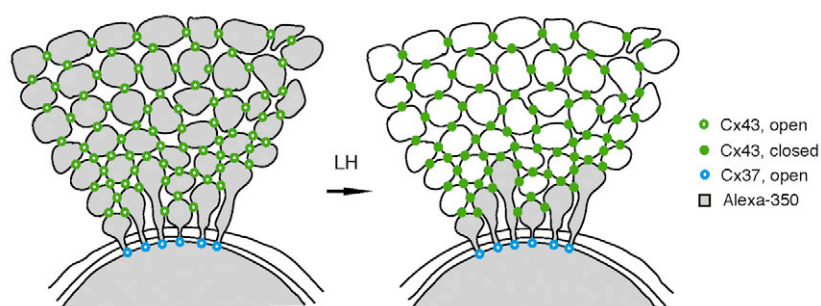
transient: the channels are closed from 0.5 to 2 hours after LH application, and then reopen. As discussed below, the closure of the gap junctions is functionally significant as being a mechanism for inducing meiotic resumption; the subsequent reopening of the junctions could be important for other processes in the follicle that might require gap junctional communication between the oocyte and somatic cells, such as the transfer of substrates for energy metabolism (see Johnson et al., 2007) or the regulation of steroidogenesis (Borowczyk et al., 2007).

Gap junction closure is sufficient for initiating the prophase-to-metaphase transition, because NEBD occurs when junction permeability is reduced experimentally. However, our results also show that an additional meiosis-stimulatory mechanism functions in parallel, as the inhibition of MAP kinase activation, which prevents the LH-induced channel closure, does not prevent the NEBD in response to LH. Thus, gap junction closure is one of two redundant mechanisms by which LH reinitiates meiosis. Much remains to be determined about both the gap junction closure-dependent and -independent pathways. In particular, what is the gap junction permeant molecule(s) required to maintain meiotic arrest, and how does the restriction of their diffusion cause meiotic resumption? Likewise, how does the gap junction closure-independent signal act to cause meiotic resumption? And finally, why has such a redundant system evolved?

It has been proposed that the mural granulosa cells produce a small molecule that passes through gap junctions into the oocyte and inhibits cAMP degradation in the oocyte, and that this molecule could be cGMP (Törnell et al., 1991). The predominant cAMP phosphodiesterase in the oocyte is PDE3A (Masciarelli et al., 2004), and PDE3A is competitively inhibited by cGMP (Hambleton et al., 2005). A role for cGMP in maintaining meiotic arrest is supported

by the findings that cGMP injection into isolated oocytes delays meiotic resumption (Törnell et al., 1990) and that treatment of rat follicles with an inhibitor of soluble guanylate cyclase causes meiotic resumption (Sela-Abramovich et al., 2008). In addition, inhibitors of inosine monophosphate dehydrogenase (IMPDH), which is required in the pathway leading to formation of cGMP, caused meiotic resumption when injected into mice (Downs and Eppig, 1987) or when applied to cultured follicles (Eppig, 1991). If gap junction closure reduced the supply of cGMP to the oocyte, this would increase the activity of PDE3A. Although such an increase was not seen in response to carbenoxolone treatment (Sela-Abramovich et al., 2006), a cGMP-mediated change in PDE activity might have been undetectable by this method, owing to the dilution of cGMP in the assay. If PDE activity did increase, the resulting decrease in cAMP would relieve the inhibition of Cdk1, linking closure of somatic cell gap junctions to meiotic resumption in the oocyte.

The concept of an alternative pathway linking LH action to meiotic resumption, which is independent of gap junction closure, and which involves a positive stimulus rather than a reversal of the mural cell inhibition, is supported by studies of isolated cumulus-oocyte complexes. Oocytes within their cumulus masses resume meiosis spontaneously, but this can be prevented by incubation with dbcAMP or the cAMP phosphodiesterase inhibitor IBMX. Under these conditions, EGF receptor stimulation, which is an intermediate in LH signaling (see Panigone et al., 2008), overcomes the inhibition imposed by dbcAMP or IBMX and causes meiotic resumption (Downs et al., 1988; Downs and Chen, 2008). Importantly, the percentage of cumulus-enclosed oocytes that resume meiosis in response to EGF is greater than that seen in isolated oocytes in the same dbcAMP- or IBMX-containing medium, implying that the



**Fig. 8. Diagram illustrating LH-induced gap junction closure.** (Left) Before LH treatment, or at 5 hours after LH application. (Right) At 0.5–2 hours after LH application.



stimulation of meiotic resumption by LH/EGF signaling results in part from a positive stimulus, in addition to the release of the mural cell inhibition.

The identity of this positive stimulus, and how it reaches the oocyte, is unknown, but it appears that the signal might pass through the Cx37 gap junctions between the cumulus cells and the oocyte, based on evidence that in the presence of the gap junction inhibitor glycylglycyl-L-homoserine, oocytes within dbcAMP-arrested cumulus complexes fail to resume meiosis in response to EGF (Downs and Chen, 2008). This finding suggests that both pathways linking LH to meiotic resumption could depend on gap junctions, although in different ways.

The functional redundancy in this regulatory system (release of inhibition by gap junction closure, as well as a positive stimulus that is independent of gap junction closure) is reminiscent of the dual pathways by which progesterone causes meiosis to resume in *Xenopus* oocytes (Haccard and Jessus, 2006). In *Xenopus*, progesterone increases the synthesis of both cyclin B and MOS, but synthesis of either protein is sufficient to cause NEBD; thus the identified redundancy occurs in the oocyte itself, rather than in the somatic cells of the follicle. Redundant signaling mechanisms occur in many other physiological and developmental processes as well, such as chemokine signaling in the immune system (Mantovani, 1999) and the specification of dorsal structures in vertebrate development (Khokha et al., 2005), and such redundancy is thought to both confer robustness and facilitate evolutionary change (Kirschner and Gerhart, 1998). The evolutionary modification of molecules required for meiosis, which might occur to optimize their functions in other tissues, could be deleterious for reproduction, so redundancy at multiple levels in meiotic signaling pathways would appear to be advantageous.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/19/3229/DC1>

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