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Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase is required for rapid resumption of oocyte meiosis in response to luteinizing hormone

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ABSTRACT

The meiotic cell cycle of mammalian oocytes starts during embryogenesis and then pauses until luteinizing hormone (LH) acts on the granulosa cells of the follicle surrounding the oocyte to restart the cell cycle. An essential event in this process is a decrease in cyclic GMP in the granulosa cells, and part of the cGMP decrease results from dephosphorylation and inactivation of the natriuretic peptide receptor 2 (NPR2) guanylyl cyclase, also known as guanylyl cyclase B. However, it is unknown whether NPR2 dephosphorylation is essential for LH-induced meiotic resumption. Here, we prevented NPR2 dephosphorylation by generating a mouse line in which the seven regulatory serines and threonines of NPR2 were changed to the phosphomimetic amino acid glutamate (*Npr2*–7E). *Npr2*–7E/7E follicles failed to show a decrease in enzyme activity in response to LH, and the cGMP decrease was attenuated; correspondingly, LH-induced meiotic resumption was delayed. Meiotic resumption in response to EGF receptor activation was likewise delayed, indicating that NPR2 dephosphorylation is a component of the pathway by which EGF receptor activation mediates LH signaling. We also found that most of the NPR2 protein in the follicle was present in the mural granulosa cells. These findings indicate that NPR2 dephosphorylation in the mural granulosa cells is essential for the normal progression of meiosis in response to LH and EGF receptor activation. In addition, these studies provide the first demonstration that a change in phosphorylation of a transmembrane guanylyl cyclase regulates a physiological process, a mechanism that may also control other developmental events.

1. Introduction

Meiosis in mammalian oocytes begins during embryonic development and is paused at prophase until, beginning at puberty, luteinizing hormone (LH) that is secreted from the pituitary gland during each reproductive cycle acts on the ovarian follicle to release the arrest. In the preovulatory follicle, meiotic arrest is maintained by cGMP that is produced outside of the oocyte, in the granulosa cells surrounding it; cGMP diffuses into the oocyte through gap junctions (Norris et al., 2009; Vaccari et al., 2009; Zhang et al., 2010; Shuhaibar et al., 2015). In the oocyte, cGMP acts by inhibiting the cAMP phosphodiesterase PDE3A, resulting in a high level of cAMP that maintains the CDK1 kinase in a phosphorylated and inactive form, thus inhibiting the prophase-to-metaphase transition (Conti et al., 2012; Holt et al., 2013). LH signaling induces meiotic resumption by lowering cGMP in the granulosa cells and, as a consequence of equilibration through gap junctions, decreases cGMP in the oocyte (Norris, et al., 2009; Vaccari et al., 2009; Liu et al., 2014; Shuhaibar et al., 2015). Thus cAMP in the oocyte decreases and meiosis resumes, initiating the series of events by which chromosomes segregate in preparation for fertilization (Schuh and Ellenberg, 2007).

cGMP in the follicle is produced by natriuretic peptide receptor 2 (NPR2), also known as guanylyl cyclase B, which is expressed in both the mural granulosa and cumulus cells, but not in the oocyte or in the theca cells surrounding the follicle (Jankowski et al., 1997; Zhang et al., 2010; Tsuji et al., 2012; Geister et al., 2013). Based on studies of mutant mice with defective NPR2, it has been established that NPR2 is essential for maintaining meiotic arrest (Zhang et al., 2010, 2011; Tsuji et al., 2012; Geister et al., 2013). NPR2 is also essential for other developmental processes including chondrocyte differentiation (Tamura et al., 2004; Bartels et al., 2004) and axon bifurcation (Ter-Avetisyan et al., 2014). It is not well

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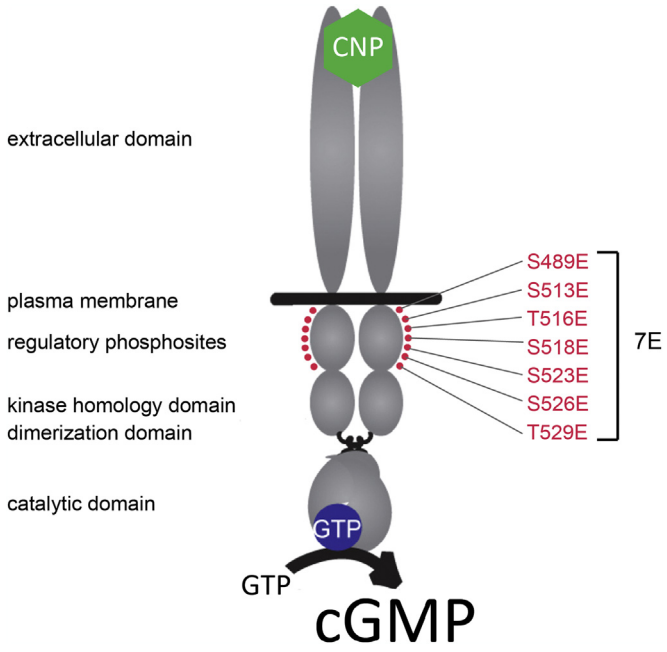

Fig. 1. Diagram of the NPR2 guanylyl cyclase showing the 7 serine and threonine phosphorylation sites that were changed to glutamates (E) in the *Npr2*–7E mice. Binding of CNP (green) to the extracellular domain and phosphorylation of 7 serine or threonine residues (red) increase catalytic activity. Dephosphorylation of these regulatory sites decreases catalytic activity; the 7E mutations result in a protein that cannot be dephosphorylated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

understood for any of these processes how other regulators, such as hormones and growth factors, are coordinated with NPR2 regulation, but recent studies of ovarian follicles have begun to identify these mechanisms (Egbert et al., 2014; Liu et al., 2014).

NPR2 is a homo-oligomeric transmembrane protein, probably a dimer, that is activated by C-type natriuretic peptide (CNP). NPR2 consists of an extracellular CNP-binding domain, a single-membrane spanning region, a juxtamembrane regulatory region, a kinase homology domain, a dimerization domain, and a guanylyl cyclase catalytic domain (Potter, 2011) (Fig. 1). Phosphorylation of serine and threonine sites in the juxtamembrane domain and the beginning of the kinase homology domain is required for CNP-dependent activation of NPR2, and dephosphorylation is a mechanism of inactivation (Potter and Garbers, 1992; Potter, 1998, 2011; Yoder et al., 2010, 2012).

LH activates a G-protein coupled receptor in the outer (mural) granulosa cells (Wang and Greenwald, 1993; Bortolussi et al., 1977; Hunzicker-Dunn and Mayo, 2015), and this leads to dephosphorylation and inactivation of NPR2 by 10 min after LH exposure, thus reducing cGMP production in mouse and rat follicles (Robinson et al., 2012; Egbert et al., 2014). The dephosphorylation persists for at least 4 h, even if LH is washed out after a brief exposure (Egbert et al., 2014; Fig. S1). More slowly, between one and 2 h after LH exposure, levels of the NPR2 agonist CNP begin to decrease, presumably further lowering NPR2 activity (Kawamura et al., 2011; Robinson et al., 2012; Tsuji et al., 2012; Liu et al., 2014). In parallel with these events that decrease NPR2 activity, the cGMP phosphodiesterase PDE5 becomes phosphorylated, which is likely to increase its hydrolytic activity (Egbert et al., 2014). In addition, LH signaling increases EGF receptor kinase activity, which by means that are not understood, contributes to the cGMP decrease and is essential for rapid resumption of meiosis in response to LH (Park et al., 2004; Norris et al., 2010; Hsieh et al., 2011; Liu et al., 2014).

The complexity of these processes raises the question of whether NPR2 dephosphorylation is essential for LH-induced resumption of meiosis. While a previous study showed that inhibiting NPR2 dephosphorylation with phosphatase inhibitors reduced the LH-induced decrease in guanylyl cyclase activity (Egbert et al., 2014), other proteins would also have been prevented from being dephosphorylated by the broad specificity phosphatase inhibitors. In particular, these inhibitors also act on phosphatases in the oocyte, causing meiosis to resume independently of LH (Rime and Ozon, 1990). Thus this approach did not provide information as to whether the rapid dephosphorylation and inactivation of NPR2 is essential for meiotic resumption in response to LH or EGF receptor activation. Here we investigate these questions using a genetically modified mouse in which the 7 juxtamembrane serine and threonine residues in NPR2 are changed to glutamates in both endogenous alleles encoding NPR2 (*Npr2*–7E/7E) (Fig. 1), such that the enzyme behaves as if it is constitutively phosphorylated. Our results indicate that dephosphorylation is necessary for NPR2 inactivation, and for the rapid resumption of meiosis in response to signaling by luteinizing hormone and the EGF receptor ligand epiregulin.

2. Materials and methods

2.1. Mice, follicle culture, and microscopy

Generation of the *Npr2*–7E mouse line, globally expressing 7 glutamate substitutions, is described in the Supplementary materials and methods and Fig. S2. The cGi500 mouse line was provided by Robert Feil (Thunemann et al., 2013). Ovaries were obtained from pre-pubertal mice (22–25 days old). All experiments were conducted as approved by the University of Connecticut Health Center Animal Care Committee.

Antral follicles (~300–400 μm in diameter) were isolated using fine forceps and were cultured for 24–30 h on Millicell organotypic membranes before use, as described by Norris et al. (2008) except that 3 mg/ml BSA was included in the culture medium in place of serum. 10 ng/ml of follicle stimulating hormone was included in the medium to stimulate follicle growth and expression of LH receptors. LH was used at a concentration of 10 μg/ml. Ovine LH and ovine follicle stimulating hormone were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). Epiregulin (R&D Systems) was used at a concentration of 100 nM. For some experiments, cumulus–oocyte complexes were isolated from the cultured follicles, by slitting them with a 30 gauge needle. Oocytes were isolated from cumulus–oocyte complexes by aspiration through a glass pipet with an approximately 80 μm diameter opening.

Observations of follicles on Millicell membranes were made using a 20x/0.4 NA long-working distance objective. LH was applied to the medium in the dish holding the Millicell membrane, and oocytes within follicles were observed for the presence or absence of a nuclear envelope and nucleolus at 1-h intervals. At 8 and 12 h after LH application, the oocyte was not visible within the follicle due to the secretion of extracellular matrix by the cumulus cells; therefore follicles were opened to isolate and observe the oocyte. Photographs were taken using a Canon EOS M camera.

2.2. Guanylyl cyclase activity assays

For guanylyl cyclase activity measurements, crude membranes were prepared and assayed as previously described (Robinson and Potter, 2011; Robinson et al., 2012). For each membrane preparation, 80–100 antral follicles from 5–8 *Npr2*–7E/7E or *Npr2*−+/+ mice were divided into 2 equal groups, and half were exposed to LH for 20 min, before preparing and freezing membrane samples

in phosphatase inhibitor buffer to preserve the phosphorylation state of NPR2. Assays were performed in the presence of 1 μM CNP, 1 mM ATP, 1 mM GTP, and 5 mM $MgCl_2$, or 1% Triton X-100, 1 mM GTP, and 5 mM $MnCl_2$, for 9 min at 37 °C. 0.5 mM IBMX was included in the assay buffer to inhibit cyclic nucleotide phosphodiesterase activity.

2.3. Western blotting and deglycosylation

Amounts of NPR2 protein were compared by western blotting, using an antibody provided by Hannes Schmidt (Ter-Avetisyan et al., 2014). To deglycosylate NPR2, 10 μg of follicle protein was incubated with Peptide: N-glycosidase F (PNGase F, New England BioLabs, Ipswich, MA) following the manufacturer's protocol. Immunodensities of western blot bands were determined using ImageJ software (http://imagej.nih.gov/ij/).

2.4. cGMP measurement

To examine the effect of LH on cytosolic cGMP, mice expressing the cGi500 sensor for cGMP (EC_{50}=500 nM) (Thunemann et al., 2013) were bred with mice from the *Npr2*–7E line, to obtain mice of either the *Npr2*–7E/7E or *Npr2*−+/+ genotype, expressing one copy of the cGi500 transgene. Follicles were imaged by confocal microscopy in a 200 μm-thick glass-bottomed chamber, before and after perfusion with LH (Shuhaibar et al., 2015). cGMP binding to cGi500 decreases FRET between CFP and YFP, such that higher CFP/YFP emission ratios after CFP excitation indicate higher cGMP concentrations (Russwurm et al., 2007; Thunemann et al., 2013). CFP and YFP images were collected every 30 s, and intensities in the mural granulosa region were measured from a 25 μm-wide band just inside the basal lamina.

2.5. Statistical analysis

Differences between treatment conditions were analyzed by unpaired or paired *t*-tests, or by one-way ANOVA with the Tukey adjustment for multiple comparisons, using Prism software (GraphPad).

3. Results

3.1. NPR2 dephosphorylation in the mural granulosa cells is required for the LH-induced decrease in guanylyl cyclase activity

To investigate if dephosphorylation of the 7 regulatory serines and threonines of NPR2 is required for the LH-induced decrease in guanylyl cyclase activity, we developed a genetically modified mouse in which the 7 regulatory phosphorylation sites of NPR2 are changed to glutamates, such that the enzyme behaves as if it is constitutively phosphorylated. Using this mouse line, we compared CNP-dependent NPR2 activity in wild-type and *Npr2*–7E/7E follicles. In untreated follicles, NPR2 activity was the same for wild-type and *Npr2*–7E/7E (Fig. 2A). However, while wild-type follicles that had been treated with LH for 20 min showed an approximately 50% decrease in NPR2 activity (Fig. 2B, left), LH caused no significant change in NPR2 activity in membranes from *Npr2*–7E/7E follicles (Fig. 2B, right). These findings indicate that dephosphorylation of these regulatory serines and threonines of NPR2 is necessary for the hormonal regulation of guanylyl cyclase activity in the ovarian follicle.

To test if NPR2 protein levels were the same in *Npr2*–7E/7E and wild-type follicles, we measured guanylyl cyclase activity in the presence of 1% Triton X-100 and 5 mM $MnCl_2$ (Fig. 2C), which

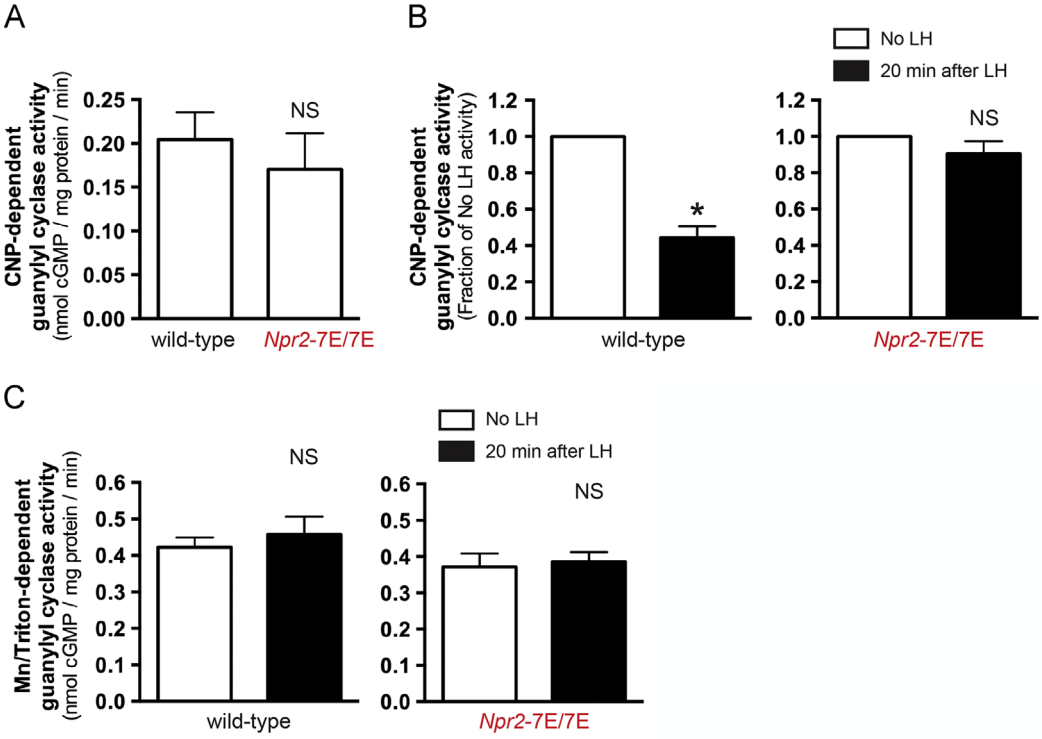

Fig. 2. Prevention of the LH-induced decrease in CNP-dependent guanylyl cyclase activity in *Npr2*–7E/7E follicles. (A) CNP-dependent guanylyl cyclase activity in membranes from wild-type and *Npr2*–7E/7E follicles, without LH treatment. (B) CNP-dependent guanylyl cyclase activity in membranes from wild-type and *Npr2*–7E/7E follicles, treated with or without LH for 20 min. Data were normalized to the no LH value for each experiment. LH treatment significantly decreased the CNP-dependent guanylyl cyclase activity in wild-type ($p < 0.05$), but not in Npr2–7E/7E follicles (NS). (C) Guanylyl cyclase activity in wild-type and *Npr2*–7E/7E follicles from the same membrane preparations as in (B), measured with 5 mM Mn^{2+} and 1% Triton X-100 in place of CNP, ATP, and Mg^{2+}, to determine the relative amounts of NPR2 protein. The activity levels in wild-type and *Npr2*–7E/7E follicles were not significantly different, and neither activity value changed in response to LH. For A–C, each value shows the mean ± SEM for 4–5 membrane preparations.

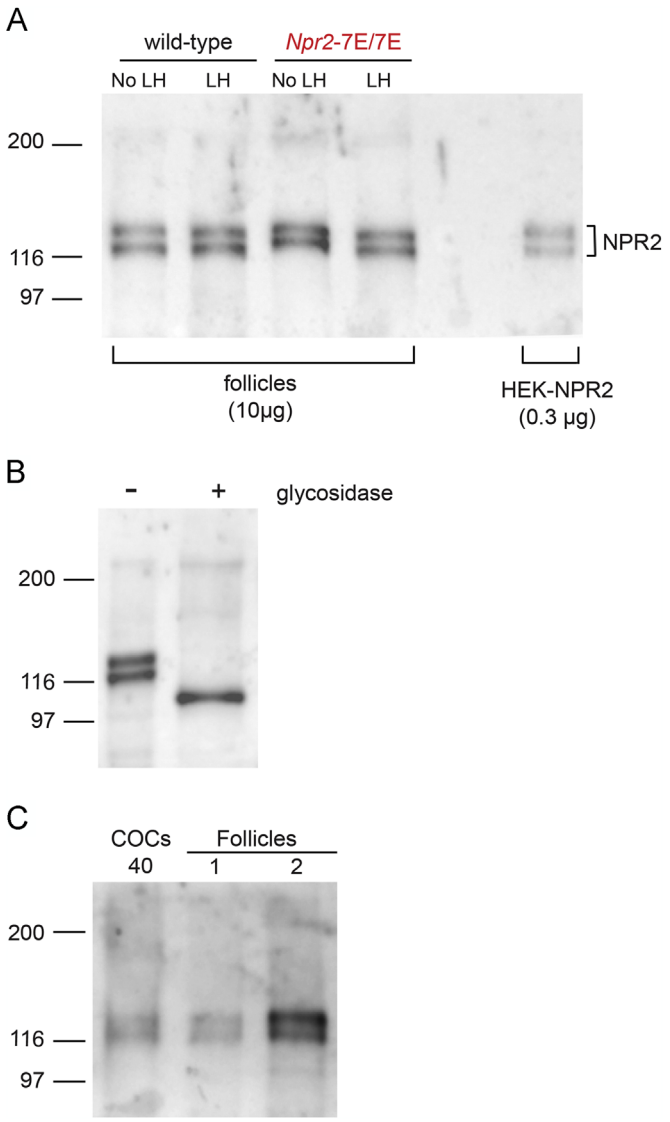

Fig. 3. Relative amounts of NPR2 protein in wild-type and *Npr2*–7E/7E follicles, with and without LH, and within different regions of the follicle. (A) Western blot indicating that the amount of NPR2 protein is the same in wild-type and *Npr2*–7E/7E follicles, and is unchanged by a 20-min LH treatment. (B) Effect of glycosidase treatment of follicle lysates, showing that the 2 immunoreactive bands correspond to NPR2 with different amounts of glycosylation (Müller et al., 2010; Amano et al., 2014). (C) Comparison of the immunodensity of NPR2 in 40 cumulus–oocyte complexes (COCs) with that in amounts of lysate from 1 or 2 follicles, showing that ~3% of the NPR2 protein in a follicle is in the COC. Since *Npr2* RNA is not detectable in the theca cells or oocyte (Jankowski et al., 1997; Zhang et al., 2010; Tsuji et al., 2012), this indicates that ~97% of the NPR2 protein is in the mural granulosa cells.

yields near maximal NPR2 guanylyl cyclase activity that is independent of CNP or phosphorylation (Potter and Hunter, 1998). Based on these assays, as well as western blots (Fig. 3A and B), neither the 7E mutations nor a 20 min LH treatment changed the amount of NPR2 protein per follicle.

Although it is known that *Npr2* RNA is present in both mural granulosa and cumulus cells (Zhang et al., 2010, 2011; Tsuji et al., 2012), the relative amount of NPR2 protein in these 2 regions has not been investigated. In some models, NPR2 protein is depicted as being located primarily in the cumulus cells (Zhang et al., 2010), which would be difficult to reconcile with our activity measurements from whole follicles. For this reason, we investigated the NPR2 protein distribution by comparing NPR2 levels in whole follicles and cumulus–oocyte complexes. Our results indicated that ~97% of the total NPR2 protein in the follicle is located in the mural granulosa cells (Fig. 3C), implying that the LH-induced decrease in NPR2 phosphorylation and activity occurs primarily in the mural cells, where the LH receptors are located (Wang and Greenwald, 1993; Bortolussi et al., 1977; Hunzicker-Dunn and Mayo, 2015).

Our results comparing NPR2 activity in membranes from follicles of *Npr2*–7E/7E and wild-type mice are consistent with measurements showing that the 7E mutations have little or no effect on substrate-binding and maximal catalytic activity of NPR2 in transfected HEK cells, but result in resistance to phosphorylation-dependent inactivation (Yoder et al., 2012). The 7E mutations also have no effect on the CNP concentration required to activate NPR2 to half its maximum value (EC_{50}) (Fig. S3).

3.2. NPR2 dephosphorylation is required for part of the LH-induced decrease in cGMP

To investigate the effect of the 7E mutations on the LH-induced decrease in cGMP levels, we used *Npr2*–7E/7E and wild-type follicles that co-expressed the cGMP FRET sensor cGi500 (Thunemann et al., 2013; Shuhaibar et al., 2015). With this sensor, the CFP/YFP emission ratio provides a measure of cytosolic cGMP. Live follicles were imaged with a confocal microscope, and CFP/YFP ratios were measured in the mural granulosa cells.

The cGi500 CFP/YFP emission ratios before LH treatment were similar for the two genotypes, indicating that the 7E mutations do not alter the basal concentration of cGMP in the granulosa cell cytosol (Fig. 4). However, in *Npr2*–7E/7E follicles, the LH-induced decrease in cGMP was attenuated (Fig. 4). Thus the dephosphorylation-mediated decrease in NPR2 guanylyl cyclase activity is an important cause of the LH-induced decrease in cGMP in the follicle, although it appears not to be the only cause, since a partial cGMP decrease occurred in the *Npr2*–7E/7E follicles. An LH-induced increase in cGMP phosphodiesterase activity might account for the residual cGMP decrease seen when LH was applied to *Npr2*–7E/7E follicles (Egbert et al., 2014).

3.3. NPR2 dephosphorylation is required for the rapid resumption of meiosis in response to LH or epiregulin

To investigate whether the LH-induced dephosphorylation and inactivation of NPR2 is required for meiotic resumption, we isolated *Npr2*–7E/7E and wild-type follicles and observed them in culture before and after addition of LH. The *Npr2*–7E/7E follicles had normal morphology, and by 8 h after stimulation with LH, the cumulus cell region underwent the normal expansion that results from secretion of a hyaluronan-rich extracellular matrix between the cells, in preparation for ovulation (Eppig, 1982; Salustri et al., 1992) (Fig. 5A).

However, follicle-enclosed oocytes from *Npr2*–7E/7E mice showed a delay in resumption of meiosis in response to LH, as indicated by a delay in nuclear envelope breakdown (NEBD) (Fig. 5B). In control wild-type follicles, NEBD occurred between 2 and 6 h after LH exposure. In contrast, in *Npr2*–7E/7E follicles, no NEBD occurred in the first 6 h following treatment with LH. The 7E mutations had no effect on meiotic resumption in response to isolating oocytes from the follicle (Fig. 5C), consistent with the lack of NPR2 expression in the oocyte (Zhang et al., 2010). Thus, dephosphorylation and inactivation of NPR2 is needed for the rapid LH-induced meiotic resumption, but not for the meiotic resumption that occurs when oocytes are disconnected from the granulosa cells that are the source of the inhibitory cGMP.

The signaling pathways leading to the dephosphorylation of NPR2 in response to LH are incompletely understood, but one likely factor is activation of the EGF receptor, since EGF receptor activity is required for rapid resumption of meiosis, and for a

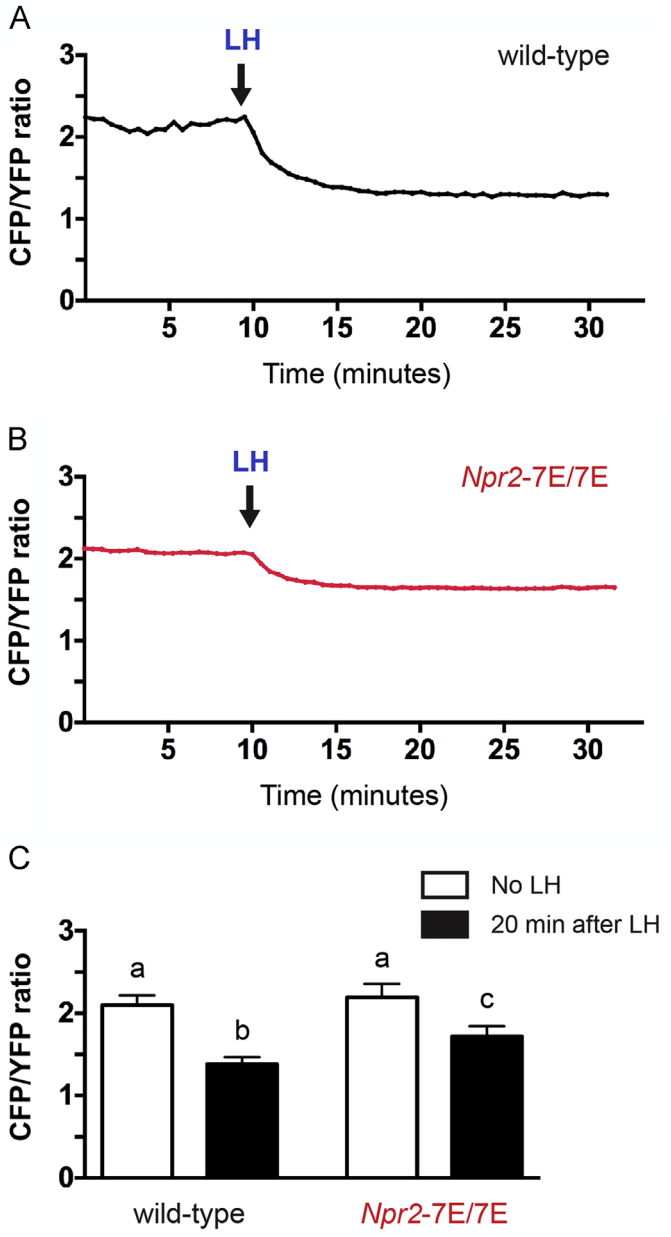

Fig. 4. Attenuation of the LH-induced cGMP decreases in *Npr2*–7E/7E follicles. Representative records showing LH-induced decreases in cytosolic cGMP in mural granulosa cells of follicles expressing the cGi500 sensor, comparing wild-type (A) and *Npr2*–7E/7E (B). (C) Measurements before and at 20 min after LH treatment. LH decreased the CFP/YFP ratio in the Npr2–7E/7E follicles to 1.72 ± 0.04 compared to 1.38 ± 0.03 in wild-type follicles (mean ± SEM, $n=9$ and 7 follicles, respectively). Values with different letters are significantly different ($p < 0.05$).

component of the cGMP decrease, in response to LH (Park et al., 2004; Norris et al., 2010; Hsieh et al., 2011; Liu et al., 2014; Shuhaibar et al., 2015). Application of the EGF receptor ligand epiregulin to isolated follicles caused meiosis to resume (Fig. 5D), and as with LH, meiotic resumption in response to epiregulin was delayed in *Npr2*–7E/7E follicles (Fig. 5D). Thus EGF receptor signaling is likely to contribute to establishing and/or maintaining dephosphorylation of NPR2.

Although meiotic resumption was delayed by the 7E mutations, it did occur eventually. By 8 h after LH application, NEBD had occurred in approximately 40% of *Npr2*–7E/7E follicle-enclosed oocytes, and in approximately 90% by 12 h (Fig. 5B). Compared to the time course of NEBD in wild-type follicles, NEBD in the *Npr2*–7E/7E follicles occurred with a 5-h delay. A similar delay was seen when epiregulin was used to stimulate meiotic resumption (Fig. 5D). Consistent with the eventual resumption of meiosis, the 7E mutations caused no obvious defect in fertility (Fig. 5E); however, further studies would be needed to determine if there is a subtle defect.

A possible cause of the eventual resumption of meiosis is the disruption of gap junction communication due to cumulus expansion (Gilula et al., 1978; Eppig, 1982); cumulus expansion occurred similarly in *Npr2*–7E/7E and wild-type follicles (Fig. 5A and F). Loss of communication between the oocyte and granulosa cells would remove the source of inhibitory cGMP and allow meiosis to resume (Norris et al., 2009; Vaccari et al., 2009).

4. Discussion

These studies contribute three new findings about how LH signaling causes meiosis to resume in the oocyte: (1) dephosphorylation of the NPR2 guanylyl cyclase is required for the rapid resumption of meiosis in response to LH, (2) dephosphorylation of NPR2 is a component of the pathway by which EGF receptor activation mediates LH signaling, and (3) most of the NPR2 protein in the follicle is located in the mural granulosa cells. These findings indicate that NPR2 dephosphorylation in the mural granulosa cells is essential for the normal progression of meiosis in response to LH and EGF receptor activation.

If LH-induced dephosphorylation of NPR2 fails to occur, as in the *Npr2*–7E/7E mice, meiosis still resumes eventually, possibly due to disruption of gap junction communication during cumulus expansion, as mentioned above. Other LH-induced changes that could contribute to the eventual resumption of meiosis in the *Npr2*–7E/7E follicles include (1) decreasing levels of NPR2 (Jankowski et al., 1997) and the RNA encoding it (Zhang et al., 2011; Tsuji et al., 2012), detectable by 3 h after LH receptor stimulation; (2) decreasing levels of the NPR2 agonist CNP, detectable after 2 h of LH treatment and continuing to decrease over the next several hours (Kawamura et al., 2011; Robinson et al., 2012; Tsuji et al., 2012; Liu et al., 2014); and (3) LH-induced activation of cGMP phosphodiesterase activity (Egbert et al., 2014). Correspondingly, under the conditions of our experiments, the 7E mutations caused no obvious defect in fertility. Whether there are subtle defects, not detected here, remains to be examined. Because meiotic progression is essential for reliable fertility, mechanisms for LH induction of meiotic resumption involve coordinated fail-safe processes, of which NPR2 dephosphorylation is only one. Thus reduction of the function of a single pathway may have little effect on fertility. For example, various mutations that result in reduced EGF receptor signaling have been reported to have only small effects (Hsieh et al., 2007, 2011) or no detectable effect (Du et al., 2004; Andric et al., 2010) on fertility; one such mutant line was reported to have highly reduced fertility, but interpretation was complicated by possible effects outside of the ovary (Hsieh et al., 2007).

Using recombinant protein expressed in HEK cells, previous studies have shown that phosphomimetic-glutamate substituted versions of transmembrane guanylyl cyclases are resistant to dephosphorylation-dependent inactivation (Potter and Hunter, 1999; Potthast et al., 2004; Yoder et al., 2012). However, it has not been previously demonstrated that this mechanism is physiologically significant. This is the first report to show that a biological signal is transduced by dephosphorylation of a guanylyl cyclase. Our studies establish that in the ovarian follicle, normal progression of meiosis in response to LH requires dephosphorylation of NPR2.

Another developmental process that could be regulated by NPR2 dephosphorylation is longitudinal growth of bones in limbs and vertebrae. Chondrocyte growth and differentiation in the growth plate is dependent on NPR2 activity (Tamura et al., 2004;

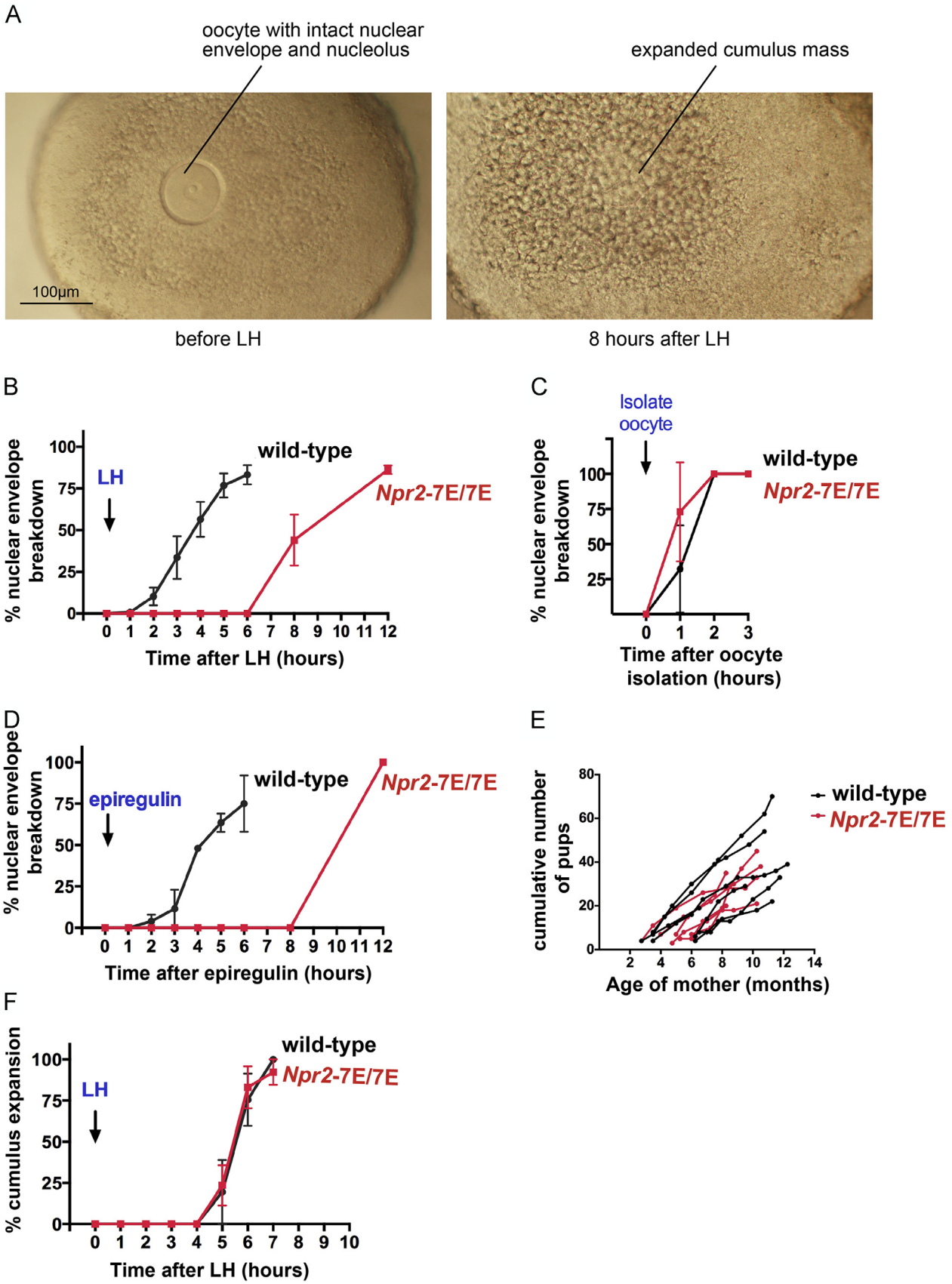

Fig. 5. Delay of LH-induced meiotic resumption in *Npr2*–7E/7E follicle-enclosed oocytes. (A) An Npr2–7E/7E follicle before LH treatment, with the oocyte arrested in prophase (left), and at 8 h after LH treatment, when the oocyte is no longer visible due to cumulus expansion (right). (B) Time course of LH-induced nuclear envelope breakdown in *Npr2*–7E/7E and wild-type follicles. (C) Time course of nuclear envelope breakdown after isolating oocytes from Npr2–7E/7E and wild-type follicles without LH treatment. (D) Time course of epiregulin-induced nuclear envelope breakdown in Npr2–7E/7E and wild-type follicles. (E) No obvious defect in fertility of *Npr2*–7E/7E mice, as judged by the number of pups produced by 7E/7E × 7E/7E breeding pairs, compared with wild-type pairs. The graph shows the number of pups produced by individual breeding pairs as a function of maternal age (7 pairs for WT, and 6 pairs for 7E/7E). (F) Time course of the initiation of LH-induced cumulus expansion, as scored by loss of a clear image of the oocyte periphery, in *Npr2*–7E/7E and wild-type follicles. B,C,D, and F show the mean ± SEM for 3–8 sets of measurements at each time point. Each set of measurements included an average of 12 follicles.

Bartels et al., 2004), and cGMP production by NPR2 in a chondrocyte cell line is decreased by FGF receptor 3 signaling (Ozasa et al., 2005), suggesting that FGF could regulate NPR2 activity by way of dephosphorylation. Axon bifurcation also requires NPR2 (Ter-Avetisyan et al., 2014), and this process could potentially be regulated by NPR2 dephosphorylation. Thus our demonstration that LH signaling regulates meiosis by way of NPR2 dephosphorylation provides a model for future investigations of regulation of guanylyl cyclases in other developmental systems.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.10.025.

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